THE METABOLIC GRADIENTS OF VERTEBRATE EMBRYOS. I. TELEOST EMBRYOS.

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I. Introduction.

The present paper is a record of observations on the disintegration gradients of some teleost embryos. Since these gradients have served to explain much that was formerly obscure in the physiology and development of organisms, it has seemed to us worth while to study them in as wide a range of living forms, both adult and embryonic, as practicable. In particular it has seemed desirable to determine the susceptibility gradients in the vertebrates in order to analyze their rôle in normal and teratological development and in the physiology of at least certain systems.[1] Since it is impracticable to study adult vertebrates by the susceptibility method owing to the difficulty of determining the time of death of different regions, our attention has been necessarily confined to embryonic stages. The recent publication of Bellamy ('19) on the frog represents the first of these investigations on the vertebrate embryo. In this paper Bellamy described the disintegration gradients of the frog at various stages of development and showed that the teratological forms experimentally produced by him and by a number of previous investigators can be easily and simply accounted for on the basis of these gradients. Similarly in these papers I shall describe the disintegration gradients of other vertebrate embryos and attempt to correlate them with well-known facts of normal and teratological development.

When an organism is exposed to a lethal concentration of a toxic solution, it is found that all of its parts do not die simul-

[1] In particular the gradients have been of great service in interpreting the physiology of the nervous system, the digestive system, and the heart.

taneously, but that they die at different time intervals. In other words, the parts of the organism exhibit a *differential susceptibility* towards the toxic substance. This differential susceptibility bears a direct relation to the organization and axiation of the organism. In the simplest cases this relation is: that the anterior or apical end is the most susceptible and dies and disintegrates first, and that the susceptibility decreases in a graded manner from the apical end along the antero-posterior or apico-basal axis. A death or disintegration gradient (so-called because the time of death is recognized by the disintegration of the part affected) is thus observable. This simple gradient has been designated by us the *primary gradient.* In more complex forms *secondary* gradients often appear after the early stages of development and may persist throughout life; they consist in the appearance of regions of high susceptibility other than the apical or anterior end. In complex animals it is further found that organs and systems may develop gradients of their own which do not necessarily correspond to or bear any relation to the more general primary gradient of the organism. As an example of an organ possessing an axiation independent of that of the rest of the body may be mentioned the vertebrate heart.

The primary gradient is initiated in protoplasm through the action of the external world upon it. Such environmental action is designated a stimulus and the point of stimulation becomes *ipso facto* the region of high susceptibility from which both the effect of the stimulus and the susceptibility gradually diminish, finally dying out. Under repeated stimulation such gradients, originally temporary, become morphologically fixed in the protoplasm to a more or less permanent degree. Since these gradients arose in response to the external world they are retained most completely and in a least altered condition in the external surface of organisms, and its derivative, the nervous system. As the internal organs and systems increase in number and complexity it is not to be expected that they would necessarily develop gradients corresponding to the gradient of the surface structures but rather that, since the gradient is the expression of physiological activity, their gradients will be the expression of their relation to the

organism as a whole and to its parts. Consequently secondary gradients are of common occurrence in complex animals.

Owing to the fact that the disintegration gradients are observable and identical in a wide range of substances of very varied chemical properties and constitutions, it is certain that they are not due to any specific action of these substances upon protoplasm. This is further evidenced by the fact that organisms display the same differential susceptibilities to extremes of physical conditions, such as high temperatures, and to low oxygen supply. Owing to the fact that differential susceptibility is directly related to physiological conditions such as age, starvation, regeneration, motor activity, stimulation, etc., it is further certain that differential susceptibility is not primarily an expression of structural or morphological gradations along the axes of organisms. All of the facts at hand lead us irresistibly to the conclusion that the gradients are physiological in nature, that they are manifestations of a quantitative gradation in function and metabolism along the axes of organisms. We are therefore accustomed to refer to these gradients as metabolic gradients; physiological gradients would possibly be a better term, as Professor Child has recently suggested.[1]

The susceptibiltiy method is thus a method for determining in a general way the relative rates of activity of different parts of the organism. Its results do not necessarily correspond to determinations of total metabolism since usually the susceptibility of only certain parts of the organism can be determined. It is not claimed that it is an accurate measure of metabolic rate, since too many factors enter into differential susceptibility, or that it should supplant methods of directly measuring metabolic rate, such as oxygen consumption, carbon-dioxide production, or determination of other metabolic end products. It has, however, served to reveal facts not discoverable by any other method at present known to us.

[1] These matters are discussed at greater length in a paper by Child now in press in the BIOLOGICAL BULLETIN and in a book "The Origin and Development of the Nervous System," shortly to be published by the University of Chicago Press. I have had the privilege of reading the manuscript of both publications and wish to acknowledge my indebtedness to them for the present argument.

In studying the metabolic gradients by the direct susceptibility method, the observer watches the time of death of different levels of the organism. It is evident that the completeness and accuracy of his observations depend on two factors: first, the ease with which the death point can be recognized, and second, the visibility of various parts of the organisms. In regard to the first matter, it may be said that the death point is usually recognized by certain changes in the appearance of the part under observation; it becomes white and opaque and loose and finally expands into a shapeless mass of granules. In the case of vertebrate embryos, owing to their delicate, almost diaphanous, structure, these changes are not always detectable with certainty, especially in very early stages, and repeated observation has often been necessary in order to make certain of the course of disintegration. As concerns the second matter, it should be perfectly obvious that the gradients can be determined only for those parts of the organism which can be seen clearly; in general these will be the superficial parts. It is usually impossible to observe the death of the entodermal structures; such is the case in the present study. The transparency of embryos, however, permits more extended observations on internal structures than is usually possible with adult organisms.

II. Disintegration Gradients of Fish Embryos.

1. *Material and Method.*—The disintegration gradients were investigated in the embryos of three species of fish—the killifish, *Fundulus heteroclitus,* the cunner, *Tautogolabrus* (*Ctenolabrus*) *adspersus,* and the cod, *Gadus morrhua.* They were obtained at Woods Hole, Mass., the first two species in June and July, and the cod in December, 1919. Sexually mature *Fundulus* and *Tautogolabrus* were obtained through the supply department and were stripped "dry," that is into vessels containing no or very little water. After fertilization had occurred water was added. In this way a high percentage of developing eggs is obtained, as previous investigators have found. The eggs of the cod were obtained from the Bureau of Fisheries at Woods Hole and I am

greatly indebted to the director of the fisheries station for his kindness in supplying me with an abundance of material. Cod eggs in all stages of development were obtainable at any time.

In studying the gradients the eggs were placed in watch glasses which were filled with the toxic solution, and observed under the low power of the compound microscope. Sometimes the watch glasses were filled full and covered with a circular piece of thin glass with the exclusion of air. It was found, however, advisable in most cases to leave the watch glasses uncovered since it was frequently necessary to turn the eggs with a needle in order to bring all parts into view. The eggs of the cunner and the cod are pelagic floating eggs but very soon after being placed in the solution they become opaque and sink to the bottom. Since the eggs in all three species rest upon the bottom of the watch glass it is easy to turn them into any desired position by mean of a needle.

For the disintegration of the embryos of Tautogolabrus, solutions of potassium cyanide in sea-water were employed, in concentrations of 1/100 mol. or stronger. When work was begun on the eggs of *Fundulus* it was immediately discovered that potassium cyanide was useless for the purpose. Apparently the egg membranes of this fish are impermeable to cyanide for the embryos will live and their hearts will continue to beat for very long periods in relatively concentrated cyanide solutions. It was found that anæsthetics and acids would penetrate the eggs of *Fundulus* very readily and stop the heart beat within a few minutes; but in none of these substances could the disintegration gradients be observed for they seemed to coagulate the embryo and it was consequently impossible to determine the time of death of different levels. Finally after fruitless trials of many substances, it occurred to me to try ammonium hydroxide, as it is well-known that this substance penetrates invertebrate eggs readily. It proved to be entirely satisfactory and was thereafter exclusively employed for the study of the gradients of *Fundulus* and the cod. In making ammonium hydroxide solutions several drops of the pure concentrated solution were added to about fifty c.c. of sea-water; the sea-water was then shaken thoroughly and

filtered to remove the abundant precipitate which is always formed on the addition of alkali to sea-water.

The drawings illustrating the distintegration of fish embryos were copied from free-hand sketches made while the embryos were under observation. They do not pretend to be accurate drawings of the embryos but are simple diagrams. The process of disintegration is represented by stippling.

2. *Disintegration of Early Blastoderm Stages.*—The very early cleavage stages were not investigated. The study began at the stage when a small blastoderm is present consisting of a number of cells. At this time in both *Tautogolabrus* and *Fundulus,* the central cells are found to be the most susceptible and from them disintegration extends to the periphery of the blastoderm. Figs. 1 and 2 show the course of disintegration in the egg of *Tautogolabrus* in an early blastoderm stage consisting of about thirty cells. The most central cells become crenated at their margins and eventually resolve into droplets; this process spreads to the periphery. Similar observations were made upon *Fundulus* eggs but they were not entirely satisfactory. In the ammonium hydroxide solutions the blastoderm of *Fundulus* invariably shrinks and arches up on the yolk. After this change has occurred, the central region of the blastoderm bursts open and subsequently disintegrates; later this disintegration extends to the periphery. These changes are illustrated in Figs. 3 and 4. The alkaline solution disturbs the normal tension existing within the blastoderm and between the blastoderm and the yolk; the blastoderm is loosened at its periphery and shrinks into a mass much smaller than normal. This occurrence has rendered it difficult or impossible to determine the course of disintegration in the early stages of *Fundulus.* It is a question whether such changes as can be observed, such as those represented in Figs. 3 and 4, are real expressions of differential susceptibility or whether they may not be due to the alterations of tension just described. In view, however, of the observations on *Tautogolabrus,* about which there cannot be any question, it is reasonable to believe that in *Fundulus* also the central region of the blastoderm is more susceptible than the periphery.

In the case of the cod, however, the state of affairs is the reverse. The earliest stages examined were those consisting of a small blastoderm composed of a considerable number of cells. At this time the periphery is more susceptible than the central portions. This is the case before the germ ring has become visible. The peripheral portion of the blastoderm becomes sharply separated from a central area and disintegrates long before the latter. This condition is illustrated in Fig. 5, the peripheral ring having disintegrated leaving a central disc still intact. In very young blastoderms, the periphery is equally susceptible at all points, as in Fig. 5, but very soon a differential susceptibility appears. One region of the periphery is found in the majority of cases to be more susceptible than any other part of the blastoderm and from this point of high susceptibility the disintegration proceeds in both directions along the periphery. Two stages in the disintegration of such a blastoderm are represented in Figs. 6 and 7. There cannot be any reasonable doubt, in view of the conditions at later stages, that this peripheral area of high susceptibility is the place at which the embryonic shield is to arise. It may therefore be stated that this region is physiologically different from the rest of the blastoderm long before its morphological rôle becomes apparent.

The metabolic conditions during the early blastoderm stages of these three species of fish are therefore of two kinds. In the case of *Tautogolabrus* and *Fundulus,* the central regions are more susceptible while in the cod the peripheral region has the highest susceptibility. There can be little doubt that these differences are the expressions of real differences in the physiology of development of the two classes of eggs. This matter is discussed further below.

3. *Disintegration of Later Stages of the Blastoderm.*—As the blastoderm of *Tautogolabrus* expands over the yolk, the region of high susceptibility comes to lie more posteriorly, that is, in that portion in which the embryo is to appear. The posterior half of the blastoderm is markedly more susceptible than the anterior half, as shown in Fig. 8. This whole region appears as far as could be determined to be equally susceptible throughout; from

this region disintegration extends forward along the margins of the blastoderm. In later stages when the germ ring is approaching the equator of the egg, the region of high susceptibility is shifted still more posteriorly. At this time the observations are somewhat obscured by the occurrence already described for *Fundulus*. The blastoderm, which covers nearly half of the egg, as shown in Fig. 9, breaks loose at its periphery and shrinks and arches up from the yolk, as in Fig. 10. Nevertheless the course of disintegration was followed in a number of individuals. A certain area along the margin of the blastoderm is more susceptible than any other region, as illustrated in Fig. 10; from this area disintegration spreads forwards and laterally as in Fig. 11. There cannot be any doubt that the region of highest susceptibility is the place where the embryonic axis is to arise. In *Tautogolabrus* by the time that the blastoderm has spread nearly half way around the yolk, the eggs float in a tilted position so that half of the blastoderm is on the upper and half on the lower side. The embryo arises in the center of the lower half. It is therefore a simple matter to determine in eggs of the stage depicted in Fig. 9 where the embryo is to arise. The place where this occurs is the region of the high susceptibility shown in Fig. 11.

Observations on the late blastoderm stages of *Fundulus* were impossible owing to the behavior of the blastoderm. It bursts at some point opposite the place where the embryonic shield is forming; it then shrinks rapidly forming a mass about the embryonic shield. In spite of repeated attempts it was impossible to come to any conclusions regarding the susceptibility relations at these stages owing to this shrinkage.

In the eggs of the cod, the germ ring is differentiated at a very early stage. The germ ring is always more susceptible than the central part of the blastoderm and one region is more susceptible than the remainder of its circumference. The disintegration is therefore the same as before the germ ring has become visible and as depicted in Fig. 6 and 7. There can be no reasonable doubt that the point of high susceptibility in the germ ring is the place where the embryonic shield originates. The shield soon

makes its appearance as a slight bulge on the germ ring as shown in Fig. 12. At this time the region of the shield is the most susceptible part of the embryo and from this region disintegration spreads in both directions along the germ ring, as in Figs. 13 and 14. As the embryonic shield grows forward its anterior margin is most susceptible and from this area disintegration extends posteriorly in the shield as shown in Figs. 15 and 16.

4. *Disintegration of the Early Embryonic Axis.*—After the germ ring has advanced half way or more over the yolk, the embryo appears in the center of the embryonic shield. Two faint lines outline its axis. In *Tautogolabrus* and *Fundulus* the embryonic shield is very faintly defined but in the cod is sharply marked out on the blastoderm. In the two first-named species, observations on the disintegration of the early embryonic axis were much obscured by the occurrence of the shrinkage already described. In but one or two cases in each species was the disintegration followed with certainty. In *Tautogolabrus* at the time when the embryonic axis first becomes visible, the course of disintegration is the following; the embryonic axis fades from view and the region in which it lies undergoes disintegration. Fig. 17 represents the normal blastoderm at this time together with the yolk; the embryonic side of the blatoderm is on the under side of the yolk. Fig. 18 shows the same blastoderm much shrunken, the yolk being omitted; the area where the embryonic axis was is seen to be in process of disintegration. As far as could be determined the whole embryonic region is equally susceptible. In *Fundulus* an early stage of the embryo was observed in but one case. In this individual two regions of high susceptibility were observed, one at the posterior end, the other at the anterior end of the embryonic axis. The first region was the more susceptible of the two; from these two points, disintegration proceeded towards the middle of the axis. Three stages in the disintegration of this individual are illustrated in Fig. 19.

Early stages of the cod embryo were observed with ease. Disintegration begins at the anterior end of the embryonic shield and proceeds posteriorly, more rapidly at the margins, as shown in Figs. 20 and 21. In a slightly later stage, the susceptibility

gradient is the same except that a secondary region of high susceptibility is faintly evident at the posterior margin of the shield. This stage is depicted in Figs. 22 to 26.

5. *Disintegration of Later Stages of the Embryo.*—After the embryo had become established its disintegration gradient was observed with ease in all three species and in a great many individuals. In *Tautogolabrus* disintegration begins at the anterior end of the embryo and proceeds posteriorly along the neural tube to its posterior end. An early embryo is depicted in Figs. 27 and 28 and a later one in Figs. 29 to 32. The disintegration gradient remains the same up to the time when the germ ring closes. The eyes are not very highly susceptible but disintegrate at about the time when the disintegration in the neural tube has extended half way back. The neural tube usually separates from the rest of the embryo and becomes arched. The time of death of the somites could not be determined with certainty as they do not seem to undergo disintegration but remain distinct long after the disintegration of the neural tube is completed. After the germ ring has closed a secondary region of high susceptibility appears at the posterior end of the embryo as shown in Figs. 33 and 34. From this time on there is no further change in the disintegration gradients; there are always two regions of high susceptibility, one at each end of the embryo; disintegration proceeds posteriorly along the neural tube and anteriorly to a slight extent from the end of the tail. In no case was the fate of the somites determined.

In *Fundulus* embryos the disintegration gradients are in general the same as in *Tautogolabrus* with certain exceptions. In *Fundulus* the two regions of high susceptibility are present from the earliest observable stages of the embryonic axis. The posterior end of the embryo is the more susceptible and disintegration begins there, and progresses anteriorly. Disintegration then begins at the anterior end of the embryo and progresses posteriorly. In the very earliest stages as in Fig. 19 this anterior disintegration begins at the tip of the neural axis. Very soon, however, the optic bulbs make their appearance. As soon as this has occurred, the optic bulbs are decidedly the most highly susceptible parts of

the anterior end. After they have disintegrated, disintegration begins at the tip of the forebrain and proceeds down the axis, meeting the disintegration progressing forwards from the posterior end in about the region of the hindbrain. An embryo of this stage, at the first appearance of the optic bulbs, is represented in Fig. 35. The first drawing in this figure shows the normal embryo, the germ ring not yet closed and somites not yet formed; the other three drawings give stages in the disintegration. Both neural tube and mesoderm are involved in the disintegration although naturally the fate of the mesoderm is less readily ascertained, owing to its loose structure.

In later stages of *Fundulus* embryos the disintegration gradients remain the same except that the secondary region of high susceptibility at the posterior end gradually decreases in importance. By the time that a number of somites have formed the eyes are the most susceptible region of the embryo. Following the eyes, the forebrain disintegrates and disintegration proceeds posteriorly. The posterior end then begins to disintegrate and disintegration proceeds slightly forwards from this region, meeting the other wave of disintegration near the posterior end of the embryo. The disintegration of an embryo of this age is shown in Fig. 36. The first drawing shows the normal embryo, the other four the course of the disintegration. The shrinkage of the embryo accompanied by a sinuous bending of the neural tube, which always occurs in later embryos in the killing solution, is also illustrated. The fate of the somites could not be observed with certainty and hence they are omitted but the high susceptibility of the segmental plate region is shown in the second drawing of Fig. 36. In still later stages of *Fundulus* embryos, the eyes are no longer more susceptible than the forebrain but both disintegrate about simultaneously. The fate of the somites could not be observed with certainty as they do not disintegrate readily. In earlier stages the somites appear to disintegrate from the posterior end forwards; in later stages when a number of somites have appeared the anterior and posterior somites seemed to be the most susceptible, the middle ones less susceptible.

The latest stages of *Fundulus* which were investigated are de-

picted in Fig. 37. The disintegration gradient is much the same as described in the preceding paragraph except that a region of high susceptibility has developed in the hindbrain where the cerebellum is forming. The end of the tail has by this time become free from the yolk and is elongating; its susceptibility is increased relative to the preceding stage. The tip of the forebrain and the eyes are about equally susceptible and from them disintegration proceeds posteriorly along the axis in both neural tube and mesoderm. The most anterior and posterior somites are more susceptible than the others and from them disintegration proceeds in both directions to the middle of the embryo. Investigation of later stages of *Fundulus* embryos was not feasible as the embryos do not disintegrate as readily as previously. It may be stated, however, that the auditory vesicles and the buds of the pectoral fins were observed to be regions of high susceptibility.

The later stages of the cod embryo resemble those of *Fundulus* except with reference to the eyes. There are always two regions of high susceptibility, the anterior and posterior ends of the embryonic axis. The anterior end commonly precedes in stages before the closure of the germ ring. Disintegration begins at the tip of the forebrain and passes backwards along the brain and eyes; next the germ ring at the posterior end of the embryonic axis disintegrates and this disintegration extends forwards; the two waves of disintegration meet anterior to the middle of the embryo. The eyes are not highly susceptible as in *Fundulus* but about as susceptible as the forebrain. This stage is illustrated in Fig. 38. As the germ ring closes the susceptibility of the posterior end increases and the eyes become temporarily more susceptible than the forebrain, as in the stage depicted in Fig. 39, where the germ ring is on the point of closure. A later stage is illustrated in Fig. 40. The susceptibility of the eyes has decreased but otherwise the disintegration gradient is the same as in the preceding stage. The disintegration of the somites as shown in Figs. 39 and 40 is the same as in *Fundulus,* the disintegration proceeding from each end to the middle. In both Figs. 39 and 40 a region of high susceptibility exists at the level

of the anterior end of the hindbrain; this appears to be related to the development of the auditory vesicles. In later stages evidences of the appearance of a region of high susceptibility associated with the formation of the cerebellum are present.

6. *The Gradient of the Fundulus Heart.*—This investigation was in reality undertaken for the purpose of studying the gradient of the heart. I was convinced from the facts known about the heart that such a gradient must exist. My observations show that such is the case; however, the matter proved more difficult of demonstration than was anticipated owing to the fact that the outer surface of the heart does not readily undergo disintegration. It was only after repeated observation that it was discovered that the disintegration processes occur only in the interior of the heart, the surface outlines remaining intact.

The gradient was studied in the *Fundulus* heart only, the hearts of the other two species of fish being too small for the purpose. The early stages of *Fundulus* proved unfavorable as the yolk sac bulges up around the embryo and conceals the heart from view. In later stages, after the heart beat is established, the disintegration gradient is invariably as follows. Disintegration begins in the wall of the sinus venosus and progresses rapidly along the heart tube to the arterial end of the heart. In this disintegration, the heart wall dissolves or melts away, leaving however, the external outlines intact; this process sweeps rapidly along the heart from the sinus to the bulbus arteriosus. This gradient was observed in numerous cases in hearts in which there was no visible differentiation along the cardiac tube. In later stages after such differentiation has occurred the gradient is more marked and steeper, that is, the disintegration passes more slowly along the heart. After the interior has disintegrated the heart tube expands but the outlines remain intact.

The gradient in the heart is shown not only by the course of the disintegration but also by the order in which the chambers of the heart cease beating in toxic solutions. In younger hearts, the order of cessation of beat is: bulbus and ventricle, auricle, sinus venosus. The sinus continues to beat feebly after the other parts of the heart have stopped contracting. The explana-

tion of this is that the heart beat originates in the sinus venosus, as has long been known, and is transmitted from the sinus in sequence along the heart tube; in order that such transmission shall occur, the original impulse must attain a certain strength. As the sinus is the most susceptible part of the heart, its beat is weakened by the action of the toxic solution and the impulse generated in it becomes too feeble to be transmitted along the length of the heart tube. At first it is able to reach as far as the auricle but not to the ventricle and bulbus, which consequently no longer contract while the auricle still continues to beat; subsequently the beat becomes too feeble to be transmitted as far as the auricle and the sinus remains beating by itself. In later stages of the heart, the auricle may continue to beat after the sinus has ceased; it is probable that as development proceeds, the auricle develops some slight degree of independence and automaticity of its own, as is well known for the auricles of the lower vertebrates, and being less susceptible to toxic agents than the sinus it may continue to contract after the latter has ceased.

The heart is by far the most susceptible part of the *Fundulus* embryo and dies and disintegrates shortly after the embryo is exposed to the ammonia solution. Older hearts are more susceptible than younger ones. This indicates that the metabolic rate of the heart increases during development.

These observations show that there is a gradation in metabolic rate along the heart tube from the sinus to the arterial end of the heart. Such a gradation is in all probability the cause of the sequence of the heart beat. The sequence of the heart beat is generally stated in textbooks of physiology to be due to the fact that the venous end of the heart possesses a more rapid intrinsic rhythm than the other chambers of the heart and consequently "sets the pace" for them. This is really only another way of saying that the venous end of the heart has a higher rate of metabolism than the rest of the heart tube, for how could it contract more rapidly than they if such were not the case? Nevertheless it does not seem to have occurred to physiologists that in such a simple gradation in metabolic rate rests the explanation of the sequence of the beat. It is a familiar physio-

logical fact that the more rapidly an organ is respiring, the more rapidly it gives off carbon-dioxide and consumes oxygen, the more rapidly does it function. A case very similar to the heart is that of the digestive tract, in which Alvarez ('18) has shown that the duodenal end of the intestine has the highest irritability, fastest respiratory rate, most rapid rate of contraction, and greatest susceptibility to drugs, of any part of the intestine and that these factors decrease along the intestine. The matter of the cause and sequence of the heart beat will be discussed more fully in later papers, as the gradient is more easily demonstrable in the chick heart.

7. General Summary of Observations on the Gradients of Teleost Embryos.—Before the appearance of the embryonic axis the central region of the blastoderm is more susceptible in *Fundulus* and *Tautogolabrus,* the peripheral region in the cod. It is highly probable that the central region would also be found to be more susceptible in the cod if a sufficiently early stage were investigated; but unfortunately this was not done. It is evident, however, that the high susceptibility of the central region, if ever present in the cod, is lost at a very much earlier stage than in the other two species. In *Tautogolabrus* (*Fundulus* being unfavorable for observations) the region of high susceptibility then gradually shifts from the central to centro-posterior regions and finally to a point on the germ ring where the embryo is to appear. In the cod also the region of high susceptibility becomes limited to the region of the germ ring where the embryonic shield subsequently develops. From this place in both species the region of high susceptibility grows forwards simultaneously with the appearance of the embryonic axis. In the embryos of all three species of fish there are sooner or later two regions of high susceptibility, the anterior and the posterior end, from both of which disintegration extends towards the middle. The posterior region of high susceptibility arises very early in *Fundulus,* later in the cod, and very late in *Tautogolabrus.* This double gradient exists in both nervous and mesodermal structures, but nervous structures are as a rule far more susceptible. A very high susceptibility of the eyes is a marked feature of *Fundulus* embryos.

Regions of high susceptibility also appear in connection with the development of the auditory vesicles and the cerebellum. A gradient exists in the heart (*Fundulus*), the venous end being the most susceptible and the susceptibility gradually decreasing to the arterial end.

III. Relation of the Gradients to Normal Teleost Development.

While it is not my purpose to enter into a detailed account of teleost development it may not be amiss to point out the bearing of the observations recorded in the preceding section on the normal course of development. In discussing this matter it is necessary to bear in mind the fact that teleost development is highly specialized. Embryologists agree in general that the primitive mode of vertebrate development is illustrated by the ganoids and amphibia, forms with total unequal cleavage. The teleost mode of development is derived from some such type and has probably proceeded along different lines in different groups of teleosts.

In the early stages of the blastoderm it was found that in *Fundulus* and *Tautogolabrus* the central cells have a higher rate of activity, as measured by the susceptibility method, than the marginal cells, while in the cod the margin of the blastoderm is more active. These facts indicate that we are dealing here with two different modes of development. In the two first-named species the germ ring and the embryonic shield are poorly differentiated and only faintly visible; in fact I have not represented them in my figures on this account. It is highly probable that in these species the germ ring plays a minor rôle in the formation of the embryo and that the embryo is produced chiefly through the activity of the central region of the blastoderm. This conclusion is supported by the observations and experiments of Morgan ('95) on *Tautogolabrus* and of Sumner ('03) on *Fundulus*. Morgan concluded that the embryo of *Tautogolabrus* is formed largely of material that has never been part of the germ ring. He also showed that the development is not affected if cuts are made in the germ ring at each side of the early embryonic

shield. Sumner concluded that in *Fundulus* the expansion of the blastoderm is centrifugal since when needles are inserted in the sides of the blastoderm the germ ring fails to expand at the points adjacent to the needles and bays in the germ ring consequently appear at those places. Similar conditions seem to be present in the salmon since according to the experiments of Kopsch ('96) the destruction of spots in the germ ring at each side of the earliest stage of the shield does not affect the formation of the embryo. It is evident that this type of development in which the center of the blastoderm is the region of high activity harks back to such a condition as that seen in the ganoids and amphibia in which the animal pole is the region of greatest activity, as evidenced by more rapid rate of division and greater susceptibility to toxic agents (Bellamy on the frog).

In the cod, on the other hand, development proceeds in a different manner. The expansion of the blastoderm over the yolk is here evidently largely due to the activity of the germ ring. In this fish as contrasted with the other two species, the germ ring becomes visible at a very early stage and it and the embryonic shield are very sharply marked off from the rest of the blastoderm. My observations further show that the margin of the blastoderm in the cod is already differentiated as a region of high activity before the germ ring is morphologically distinguishable. The mode of development of the cod by peripheral expansion may be regarded as a specialization from the more primitive type exhibited by *Tautogolabrus* and *Fundulus* and probably represents a short cut in development with omission of the original centrifugal method of growth.

With the expansion of the blastoderm we next observe in *Tautogolabrus,* a shifting of the region of high activity from the central region of the blastoderm to the central-posterior and finally to a definite region of the germ ring where the embryo is to form. It seems to me probable that in these changes in the *Tautogolabrus* blastoderm we have an illustration of the manner in which the centrifugal method of development is transformed into the germ ring type. The conditions in *Tautogolabrus* thus eventually come to be identical with those very early present in

the cod. The cod has evidently omitted the early stages and very soon arrives at the condition in which the posterior median region of the germ ring is the center of activity. There has thus been produced the typical teleost method of development by means of the embryonic shield.

In vertebrates like the frog having a primitive mode of development it is important to note that a region of high activity also develops in the median posterior point of the 'germ ring,' that is to say, the dorsal lip of the blastopore (Bellamy, *loc. cit.*). The frog, however, also retains the region of high susceptibility at the animal pole, so that two regions of high activity are constantly present—the animal pole and the dorsal lip of the blastopore. In the teleosts the former region either is not present from the first or first develops and is then lost; but subsequently a new region of high activity develops at this point. It seems therefore that the germ ring type of development is highly specialized and the attempt to interpret the modes of development of other vertebrates in terms of the germ ring is a mistaken effort; but rather germ ring types should be interpreted as modifications of the method illustrated by the amphibia.

After the region of high activity has become limited to a median posterior area of the germ ring, it extends forwards and the embryo appears in its center. That the germ ring is actively concerned in this extension appears improbable since the anterior end of the shield in the cod and all of the shield except the place where it meets the germ ring in the cunner are the regions of high susceptibility. If the forward growth were produced by the germ ring, by a sort of pushing process, then one would expect the posterior end of the shield to exhibit the highest susceptibility. It seems probable that the shield grows forwards through the activity of its anterior end or possibly in some species with the aid of the surrounding cells of the blastoderm. The conditions are evidently variable in different species. According to Kopsch ('96) if the embryonic shield of the salmon embryo is destroyed the germ ring continues to close but no embryo is formed. Here evidently the embryo arises solely from the material of the shield. In *Tautogolabrus* and *Fundulus*, however, the

cells around the shield probably take part. Thus Morgan ('95) found that in *Tautogolabrus* the embryo is formed by a concentration of material towards the center of the shield. Sumner ('03) states that if the embryonic shield of *Fundulus* is destroyed a new embryonic shield is regenerated. My observations also show that in *Tautogolabrus* and the cod the region about the shield takes part in the formation of the embryo since a large area of high susceptibility is present in the early stages, as seen in my Figs. 18 and 20 to 26. While materials around the shield may contribute to the embryo the work of Kopsch, Morgan, and Sumner agrees in denying such a rôle to the germ ring, in the formation of the early embryonic axis. Cuts or injuries in the germ ring at the sides of the shield do not affect the formation of the embryo but this proceeds in practically normal manner.

Although according to these lines of evidence the germ ring plays no rôle or only a very minor one in the early development of the embryo, it sooner or later becomes involved in the formation of the embryo. This is shown in all three species by the appearance of a region of high susceptibility at the posterior end of the embryonic axis. This active region arises very early in *Fundulus* and this fact indicates that the greater part of the *Fundulus* embryo is laid down by this posterior growing region. This was also the conclusion reached by Sumner since he found that piercing this growing region inhibits the formation of the posterior part of the embryo. This region grows backwards adding to the embryo in front of it, exactly as in the case of the primitive streak of the chick, with which it is no doubt homologous. In the cod the posterior growing region arises somewhat later; and in *Tautogolabrus* very late. In the latter species it is evident that very little of the axis of the embryo is due to the activity of this posterior region and that the embryo elongates considerably without it, apparently through growth at its anterior end. This independence of the embryo of *Tautogolabrus* of the germ ring was already noted by Morgan in 1895 since he found that by placing the eggs in diluted sea-water it was possible to retard or prevent the formation of the embryo without affecting the growth or closure of the germ ring. Experiments on other species are

unfortunately lacking except in the case of the salmon, in which according to the experiments of Kopsch, most of the trunk and tail of the embryo are produced through the activity of the portions of the germ ring immediately adjacent to the early embryonic shield. It seems in view of the facts at hand legitimate to draw the conclusion that in different teleost embryos the amount of material contributed to the formation of the embryo by the germ ring is variable. At one extreme are cases like *Tautogolabrus* in which very little material is so contributed and at the other extreme the case of *Fundulus,* where most of the embryo is formed from a growing point in the germ ring. The disagreement among investigators concerning the mode of formation of the teleost embryo is evidently due to the circumstance that they worked with different species. The mode of development in different species is not identical but rather exhibits various degrees of modification from the primitive vertebrate type illustrated by the ganoids and the amphibia to the extremely specialized type in which the germ ring plays the dominant rôle. Owing to this fact generalizations cannot be drawn from the study of a single species and controversies which have arisen in the past concerning the mode of origin of the teleost embryo are without point.

While it may be said that the evidence indicates that the germ ring does add to the embryo in varying degrees in different species, the facts recorded in this paper do not seem to me to support the theory of concrescence. The embryo is not produced by a concrescence of two areas of the germ ring even in cases like the cod where the germ ring plays an important rôle in development; but there is present in the germ ring at the posterior end of the embryonic axis a region of high activity which in some species, like *Fundulus,* is of great importance in the formation of the posterior part of the embryo. Some of the material of the germ ring passes into the embryo but not in the manner required by the concrescence theory; and the anterior end of the embryo is not formed in this way but by an independent region of activity.

The existence in the development of vertebrates—it is now known to occur in the frog and chick as well as in teleosts—of two regions of high activity, one at each end of the axis, is correlated with the fact that vertebrates are segmented animals. This mode of development is common, as far as our investigations go, to all segmented animals. This "double gradient," as we call it, was first discovered in the annelids (Hyman, '16, and Child, '17). It appears in an early stage of development in annelids and persists throughout life in all of them, so far as tested. It also appears, as we have seen, in the vertebrate embryo and persists as long as the posterior end continues to develop and elongate. In the annelids where new segments form continually throughout life, the posterior region of high susceptibility is permanent and never brought under complete control of the anterior end; but in the vertebrates it eventually dies away and segment formation thereupon ceases. The posterior region of activity is therefore correlated with the process of segment formation. There can be little doubt that segments represent incomplete individuals. As Child ('15*a*) has shown, the anterior end of an organismic axis is dominant over a certain length of the axis; beyond this level physiological isolation occurs, the metabolic activity increases, and new individuals arise. A similar process is at the basis of segmentation. The posterior growing region of the embryos of segmented animals has escaped from the control of the anterior end; it is physiologically isolated, develops a high metabolic rate and proceeds to the formation of new but incomplete individuals, that is, segments. This process of segment formation will continue indefinitely if the anterior end fails to regain control of the entire length of the axis as in the annelids but will cease when this occurs as in the vertebrates and probably arthropods. These matters have been discussed at greater length by Child ('17) and Bellamy ('19).

IV. Respiratory Rate During the Development of Fundulus.

I have made some measurements of the rate of oxygen consumption and the rate of carbon-dioxide output during the de-

velopment of *Fundulus heteroclitus*. Similar experiments had previously been performed by Scott and Kellicott ('16), but apparently no report of these experiments beyond the abstract referred to has ever been published. In this abstract it is stated that during the early stages of *Fundulus* up through the formation of the embryo the rate of oxygen consumption is less than 0.10 c.c. per hour per thousand eggs. This statement is in general true; however, it misses the really fundamental point about the respiratory rate during the early stages. I do know why the marked change which occurs at the time that the germ ring is near the equator of the egg should have missed the attention of these investigators; but at least no mention of this is made in their abstract. They noted a marked increase in rate of oxygen consumption when the heart begins to beat; beyond this time there is no marked rise but a general upward trend with a great increase after hatching.

Table I.

Rate of Oxygen Consumption of the Eggs of Fundulus heteroclitus During Development.

Results given in cubic centimeters of oxygen consumed per 1000 eggs per two hours in exps. 1, 2 and 3; per 3 hrs. in no. 4. Temp. 20° C.

No. of Experiment		1	2	3	No. of Experiment.		4
Time Since Fertilization.	State of Embryo.	Oxygen Consumed.			Time Since Fertilization.	State of Embryo.	Oxygen Consumed.
4–6 hrs.	2–4 cells	0.07	0.09	0.08	2–5 hrs.	to 8 cells	0.04
6–8 hrs.	32 cells	0.07	0.06	0.07	9–11 hrs.	small disk	0.06
9–11 hrs.	many cells	0.09	0.09	0.07	26–29 hrs.	disk $\frac{1}{3}$ over yolk	0.19
22–24 hrs.	large disk	0.09	0.09	0.08			
30–32 hrs.	far over yolk	0.12	0.13	0.15	34–37 hrs.	embryo with eyes	0.21
2 days	embryo present	0.14	0.13	0.14			
					$2\frac{1}{2}$ days		0.22
3 days		0.10	0.10	0.10	$3\frac{1}{2}$ days	circulation	0.36
4 days	good circulation	0.17	0.18	0.17	4 + days		0.27
					$5\frac{1}{2}$ days		0.25
6 days		0.15	0.15	0.14	later (average)		0.30
Later (average)		0.22	0.23	0.24			

My determinations of the rate of oxygen consumption were made by the same method used by Scott and Kellicott. The carbon-dioxide output was determined by the phenolsulphonthalein method. Forty eggs of approximately the same stage of

development were placed in tubes with the phenolsulphonthalein solution in sea-water. The pH of the sea-water at Woods Hole was found to be 8.2 by comparison with the set of standardized tubes put out by Hynson, Westcott, and Dunning. The length of time required for the forty eggs in a closed tube to turn the indicator from pH 8.2 to pH 7.6 was recorded at different stages of development. This furnishes a rough measure of the relative rate of carbon-dioxide output at successive stages. The temperature was of course kept constant as nearly as practicable throughout such experiments.

The data on the oxygen consumption are given in Table I. Four experiments were run, each consisting of over a thousand eggs mixed from a number of females. Three of these experiments were run simultaneously, the fourth one at a later time.

The experiments recorded in Table I. show that the oxygen consumption remains about the same through the early cleavage although a slight rise probably occurs. By the time, however, that the blastoderm has spread one third or half way over the yolk a marked rise in the rate of oxygen consumption occurs. In experiment 4 this rise was over 200 per cent., less in the other three experiments. From this time on through the establishment of the embryo the rate remains about the same and may even fall again. Thus the formation of the embryo is not a period of increase in the rate of oxygen consumption but rather the time of high respiratory activity is that period when the germ ring is approaching the equator of the egg. This probably corresponds to the time of gastrulation. After the heart has begun to beat the oxygen consumption rises again as also found by Scott and Kellicott. Beyond this time the determinations yielded rather irregular results. I cannot verify the statement of Scott and Kellicott that there is a general upward trend during these later stages; I found a considerable variability in the amount of oxygen consumed; in general it was very little if any higher than the rate at the time the heart had begun to beat vigorously. In the table the average of these later determinations is given. It should be emphasized that the determinations during later stages are probably unreliable owing to the growth of bacteria on the

eggs. Although the eggs were frequently washed, particularly in experiment 4, this source of error was probably present. It tends of course to make the oxygen consumption appear too great. In experiment 4 in which the eggs were thoroughly washed twice daily, the results are probably more reliable than in the other three experiments; and in this experiment no increase was observed in later stages.

These experiments lead us to believe that the rate of oxygen consumption in the development of *Fundulus* is highest at the time when the germ ring is in the neighborhood of the equator, early on the second day of development. It is probably actually highest per unit weight of protoplasm since from that time on the amount of protoplasm increases greatly but the oxygen consumption does not increase in like proportion; in fact, a considerable part of the oxygen consumption after the third day is due to the activity of the heart. As the embryo is continually increasing in size after this time while the oxygen consumption shows relatively little increase we may reasonably conclude that the oxygen consumption of the embryo per unit weight is actually decreasing. In other words, senescence is already in progress.

The study of the carbon dioxide production yielded similar results. The carbon-dioxide production per unit time increased up to the early part of the second day of development after which it fell, rising again in later periods.

This result, that the metabolic activity of the embryo is at its highest point at the period when the germ ring is near the equator, is in harmony with and furnishes an explanation of previously known facts. Child determined the susceptibility of the eggs of *Fundulus* to phenyl urethane ('15*b*, p. 416). He found that the embryos are killed more quickly at this stage than at any other stage. Since susceptibility is, as I have pointed out in the introduction, a measure of metabolic rate, this result of itself shows that the metabolic rate is highest at that period. My experiments confirm this result of Child's and further illustrate the reliability of the susceptibility method as a measure of rate of activity. Various investigators, as Stockard and Kellicott, whose work is considered at greater length later, have noted that

the *Fundulus* embryo is most affected by reagents when the germ ring is near the equator of the eggs and yields the maximum number of teratological forms at this period. Newman ('15) found that in heterogenic fish hybrids development very frequently stops at this stage.

V. Relation of the Gradients to Teratological Development.

The literature on the structure, occurrence, and experimental production of teratological vertebrate embryos has now attained such vast proportions that an adequate review of it would be a huge task. I shall here consider only the experimental production of terata among teleosts. The discussion applies, however, to vertebrate terata in general, since the mode of development is much the same throughout the vertebrates.

Morgan ('95) found that if the developing eggs of *Tautogolabrus* are placed in diluted sea-water, the development of the anterior end of the embryo is commonly inhibited; the medullary folds fail to close and the anterior end remains flattened out on the yolk. In one case the formation of the embryo was completely inhibited but the germ ring continued to develop and close in normal fashion.

The experiments of Stockard ('06, '07, '09, '10) on the production of teratological forms in *Fundulus* are familiar to every one. His first experiments were performed with lithium chloride. In the stronger solutions, the eggs either cease to develop in an early blastoderm stage or else very abnormal embryos are produced with poorly developed anterior and posterior ends, short bodies, and no eyes. The trunk and auditory vesicles are, however, present. In weaker solutions of lithium chloride, the embryos are less abnormal. The expansion of the blastoderm over the yolk may be retarded resulting in spina bifida. The embryos are commonly short with no eyes or defective eyes. The developing eggs were found to be most sensitive to the treatment between 18 and 22 hours after fertilization when the germ ring is near the equator of the egg. In later experiments Stockard found that a number of substances would produce the same effects on the

Fundulus eggs. He used potassium chloride, lithium nitrate and sulphate, calcium chloride, ammonium chloride, and magnesium chloride, alone or in combinations. In solutions of all of these substances embryos were obtained with poorly developed heads and eyes, or with no eyes, with abnormal hearts and defective circulatory systems, with shortened bodies, and open blastopores. In magnesium chloride in particular, fifty per cent. of embryos with various eye defects were obtained. All degrees of approximation of the eyes were noted, to the cyclopean condition. Many one-eyed monsters were obtained, in which one eye was small or defective or wanting. Associated with the approximation of the eyes was often an abnormality of the anterior part of the head resulting in displacement and elongation of the mouth which projected ventrally like a proboscis. The forebrain in these embryos with abnormal eyes may be nearly normal or reduced; it is always reduced when the cyclopean eye is reduced and defective. In the extreme cases, the olfactory pits were fused also. Later Stockard found that similar conditions could be produced by anaesthetics, except that the eye defects were then accompanied by other defects while with magnesium chloride it is possible to produce defective eyes in embryos otherwise nearly normal. The embryos produced in anæsthetics in addition to defective eyes nearly always have narrow and defective brains, abnormal ear vesicles, and defective posterior ends in the form of spina bifida.

Other investigators have obtained similar results. McClendon ('12*a* and *b*) obtained cylclopic *Fundulus* embryos by means of a number of salts, anæsthetics and alkaloids. He states that in nature cyclopic trout embryos arise in water containing an insufficient quantity of oxygen and that he has observed cyclopean smelt embryos which were possibly caused by an excessive carbon-dioxide content. Gee ('16) obtained abnormal *Fundulus* embryos similar to those of Stockard by alcohol and sodium hydroxide. These embryos were characterized by defective heads and eyes, asymmetrical eyes, absence of eyes, shortened bodies, defective circulation, and spina bifida. Gee found that the defects are obtained if the egg is exposed to the solutions before fertilization or shortly after fertilization. Kellicott ('16)

obtained numerous defective forms in *Fundulus* by exposing the eggs to low temperature at various periods after fertilization. The eggs do not develop while in the refrigerator but if removed even after a number of days to room temperature, some of them will develop and numerous abnormalities are produced. Although it is stated by Kellicott that every possible abnormality arises under these conditions, yet perusal of his data show that the abnormalities are in fact limited to certain parts of the embryos. These are: absent or defective head, absent or shortened tail, various abnormalities of the brain and eyes, abnormalities of the heart or circulatory system, abnormalities of wandering cells and their products.[1] It is evident that the terata obtained by Kellicott fall under the same heads as those obtained by Stockard and others. Kellicott noted a marked susceptibility to low temperature at the time when the germ ring is approaching the equator. The low temperature used by Kellicott is more effective than the chemical solutions employed by others since it greatly inhibits the development without at the same time destroying the blastoderm. It is important to note that when such greatly inhibited living masses are restored to room temperature suggestions of organs develop such as "brain fragments, lenses, portions of optic cups, groups of somites, masses of erythrocytes, rhythmically contractile cells arranged either as flat sheets or tubular hearts, scattered pigment cells of the usual types, endothelial cells over the surface of the yolk, fragments of notochordal tissue." Kellicott did not notice the fact that these fragments which develop from eggs retarded in early stages concern exactly the same parts of the embryo in general as fail to develop when the eggs are inhibited at later periods in their development. Loeb ('15) obtained blind *Fundulus* embryos by means of potassium cyanide solutions and low temperatures. One embryo is figured by Loeb which possesses eyes and tail and nothing else. Werber ('16) again obtained the same teratological types with butyric acid and acetone-embryos with defective heads, including brain (forebrain), mouth, and eyes, with approximated olfactory pits,

[1] No observations were made by me on the wandering cells of the yolk sac. It is reasonable to believe, however, that such cells are cells of high physiological activity and hence highly susceptible to toxic agents.

defective auditory vesicles, defective or absent tails. Werber also noted the same fact which as been mentioned in connection with Kellicott's experiments, namely, that in some cases, the parts which are usually inhibited may alone survive, the rest of the embryo having disappeared. Such isolated parts are the anterior end of the embryo and the eyes. In some cases the only differentiated parts of the embryo were a fragment of the brain with an eye attached.

Similar terata can also be produced by hybridization. Such terata in *Fundulus* hybrids were described by Newman ('08, '17) and Loeb ('15). Newman showed that there is a correlation between the rate of development of such hybrids and the degree of abnormality. Those which develop most slowly showed the most pronounced abnormalities. The terata are of the same types as those already described, consisting of defective and inhibited heads, brains and eyes, defective hearts, shortened bodies, as well as types in which the head and eyes alone are present.

It is highly significant to note that similar terata can be obtained by treatment of the sperm alone. Oppermann ('13) obtained them from normal eggs of the salmon fertilized by sperm which had been exposed to radium and mesothorium. The embryos resulting from such fertilizations show all of the typical defects—distortions and marked inhibition of the forebrain and eyes and general anterior end of the body, defects or inhibitions of the tail, spina bifida, some abnormality of the myotomes. Embryos were frequently obtained having neither definite heads nor tails, but only trunks. G. and P. Hertwig ('13) treated the sperm of *Gobius jozo* with methylene blue and methyl green and observed that eggs fertilized by such sperm produce abnormal embryos with defective anterior and posterior ends.

From this consideration of the literature it is obvious that a large variety of agents and conditions produce the same defects in fish embryos. These defects are primarily concerned with the following parts of the embryo: the forebrain, the head in general, the sense organs, especially the eyes, the heart and circulatory system, the tail.

The explanations of these defects have been almost as numer-

ous as the investigators concerned. These explanations have in general proved inadequate and unsatisfactory and fail to account for the facts. The most obvious explanation, proposed at first by Stockard, that the defects are the consequence of a specific action of the chemicals employed upon the embryo, was later abandoned by him and must be regarded as untenable. The fact that a large number of substances and conditions call forth the same defects at once shows that their action must be a very general one and not at all specific. The osmotic pressure of the solutions cannot be the effective factor, since solutions of varying osmotic pressure yield similar results. McClendon's proposal that the solutions alter osmotic conditions in the egg by changing the permeability of the surface cannot be accepted in view of the fact that the same defects are produced by injuring the sperm only and keeping the eggs in normal sea-water. Stockard's final conclusion that the defects are due to a general depression of the eggs by the agents to which it is exposed contains part of the truth but fails to account for the fact that only certain parts of the embryo are affected. Werber believes that the defects are due to a blastolytic destruction or dispersal of the embryo; but outside of the fact that such blastolysis cannot be demonstrated the theory fails like the others to account for the differential action of the effective agents on the embryo. Kellicott sought the explanation in a disturbance of the normal organization of the egg with abnormal arrangements and distributions of the egg materials. This theory likewise does not account for the differential effect on the embryo.

It is perfectly obvious that the outstanding fact which must be taken into consideration is that all of the reagents and conditions affect some parts of the embryo more than they do other parts. These affected parts have already been enumerated. It is quite impossible to account for this except on the assumption that certain parts of the embryo are more susceptible to alterations of conditions than other parts. The necessity for this assumption has been recognized clearly by Stockard, McClendon, and Werber, but it does not seem to have occurred to them that when this assumption is granted no further explanation is neces-

sary. It is of itself the explanation. The defects are due not to the agents used, except in a general way, but to the metabolic conditions in the egg and embryo.

The work of Child and his students upon the susceptibility gradients of organisms has shown that in fact some parts of the organism are more susceptible to external agents than others. *The differential susceptibility required to explain teratological development is then no longer an assumption but a demonstrated fact.* In the sea-urchin ('16) and in annelids ('17) Child showed that the development could be controlled and modified on the basis of the susceptibility gradients and predictable types of terata experimentally produced. A similar demonstration of the relation between the susceptibility gradients and the teratological development was made by Bellamy on the frog.

The relation between the susceptibility gradients and the production of terata is the following: Those parts of the egg or embryo having the highest susceptibility and metabolic rate are the most strongly affected by altered conditions of a depressing nature and the most greatly inhibited by them, providing that the circumstances do not permit of recovery or acclimation. On the other hand if the circumstances do permit of such recovery and acclimation than those same parts which under more severe conditions succumb are able to recover and continue to develop while parts of lower metabolic rate cannot.

In order to apply these conceptions to any particular organism it is first necessary to study the metabolic gradients in that organism. This I have done in the case of the teleost fishes and I have shown that the most susceptible parts are the forebrain, the eyes (particularly in *Fundulus*), the heart, the posterior end, and to a less extent the other sense organs.[1] It will be obvious without

[1] No observations were made on the susceptibility of the olfactory pits but in the frog Bellamy noted that they are regions of high susceptibility. In general it may be said of the sense organs of the head, that the eye is the most susceptible, the ear vesicles next, and the olfactory pits last. It is therefore possible to obtain defective eyes in embryos otherwise fairly normal but defective ear vesicles and approximated olfactory pits occur only in embryos otherwise considerably abnormal. As the matter is not discussed in the text a word may be said here about the cerebellum. The high susceptibility of the

further discussion that these parts of the embryo, shown by me to be the most susceptible to toxic agents, are also the ones showing the most defective development in the experiments which have been quoted. In all of these experiments it is evident that the agents used are inhibiting or depressing agents because as stated by the authors the development of the eggs subjected to them is slower than that of the control. Under such depressing conditions the parts with the highest susceptibility or, in other words, highest metabolic rate, will be inhibited while other parts develop; and this is actually the fact. On the other hand, if the circumstances permit, such parts can recover more readily than others, and these same parts may be found developed while other parts have succumbed. This explains the development of small parts of the embryo described by Kellicott, Werber and others—isolated eyes, hearts, fragments of brain, etc.

The susceptibility gradients therefore furnish a basis for the explanation of teratological development. No other conception which has been advanced does so serve to account for all of the facts. In particular it seems to me impossible on any other basis to explain the production of the same terata in eggs fertilized by injured sperm or by foreign sperm or in cases where the egg is treated before fertilization as in Gee's experiments. In such cases a general lowering of the metabolic rate of the egg as shown by its slower development has occurred and this could produce specific terata only in case certain parts of the embryo require a higher metabolic rate for their expression than others.

The application of the metabolic gradient conception to vertebrate teratology has already been made by several investigators. Werber ('16) recognized its bearing on the teratological *Fundulus* embryos which he produced but failed to grasp the full significance of the conception and failed to see that it rendered his own conception of differential blastolysis superfluous. Newman ('17)

cerebellum is interesting in view of the fact recognized by neurologists that the cerebellum is a supra-segmental structure added on to the brain stem in the course of evolution; and the further fact, discovered by MacArthur and Jones ('17), that the cerebellum respires about as rapidly as the cerebral hemispheres, both respiring more rapidly than other parts of the central nervous system.

clearly saw that "the principles enunciated by Child serve to rationalize the results of heterogenic hybridization." He gave what is for the most part the correct explanation of the terata originating in his hybridization experiments but fell into a number of errors because little was then known about the metabolic gradients in these fish embryos, and he assumed them to be like those of the flatworms.[1] The most complete analysis of vertebrate teratology which has been made is that of Bellamy ('19) on the frog, since in this case both the metabolic gradients and the terata resulting from the differential action of external agents on the eggs are known.

I have now shown in a general way how the terata produced experimentally in teleost embryos can be explained on the basis of the metabolic gradients. Such terata are of two general types, those due to differential susceptibility, in which the parts of highest activity are inhibited and defective, and those due to differential recovery or acclimation, in which the parts of highest activity alone survive. A more detailed discussion seems to be unnecessary in view of the extensive treatment of the matter in the papers of Child, Newman, and Bellamy already cited. I may, however, as an illustration of the application of the susceptibility results to a specific organ take the case of the *Fundulus* eye. It happens that in *Fundulus,* as I have shown, the eyes are very susceptible with reference to other parts of the body, more so than in other species of fish. This indicates that the region from which the eyes arise must be one of very high activity, and as the data of Stockard show this region must be affected before the eyes appear in order that defective eyes result. Consequently inhibition of this region results first in approximated and later in defective eyes. Now since it happens that in *Fundulus* this region is so much more susceptible than in other forms, the occurrence of eye defects in *Fundulus* will also be more common than in other forms and further it is possible to obtain eye defects

[1] In particular the statements made by Newman about the gradient of the heart and circulatory system are quite erroneous. Further the posterior end of fish embryos is a region of high metabolic rate and embryos with defective posterior ends are probably due to direct inhibition and are not recovery types as supposed by Newman.

in embryos otherwise nearly normal, if the inhibiting agent is a rather weak one. This is not possible in other forms; I venture to predict that such a result could not be obtained in Tautogolabrus but that defective eyes in this species would always be associated with marked defects of the brain and other parts of the head. Such is the case in the frog, where cyclopic eyes occur only in markedly microphthalmic embryos. Owing also to the high metabolic rate of the *Fundulus* eye it is possible in this form for the eye to recover and survive when nearly all other parts of the embryo are killed. I also venture to predict that the occurrence of such isolated and solitary eyes in the absence of other parts of the embryo will be found to be rather rare and unusual in other species.[1]

In conclusion I may reiterate that the study of the metabolic gradients such as has been made in this paper furnishes a rational basis for the understanding and interpretation of normal and teratological development. While the particular organism which is to develop from a given egg is determined by the hereditary constitution of that egg, the orderly sequence of development, the spatial relations and proportions of parts, and the general axial organization are controlled by physiological, metabolic differences between different parts of the developing egg. Such physiological differences arise in the final analysis through the action of external conditions on protoplasm. By modifying in a purely non-specific, quantitative manner the metabolic differences at different levels, orderly predictable departures from the normal course of development are obtainable.

VI. Summary.

1. The susceptibility of developing eggs of *Fundulus,* the cunner and the cod to toxic solutions at various stages was studied.

2. In early blastoderms the central region is more susceptible in *Fundulus* and the cunner, the peripheral region in the cod.

[1] Monopthalmia, often observed in *Fundulus* embryos, is simply due to a greater susceptibility of one side than the other; the eye on the more susceptible side is inhibited. In the course of my studies on vertebrate embryos, this asymmetrical susceptibility has frequently been noted although the figures are drawn as if the susceptibility were always bilaterally symmetrical.

3. In late blastoderms, the median posterior region of the germ ring where the embryonic shield is to arise is the most susceptible region.

4. After the formation of the shield, its anterior portion is the most susceptible.

5. After the origin of the embryonic axis the anterior end of the axis is the most susceptible and from this point the susceptibility decreases posteriorly.

6. Sooner or later a secondary region of high susceptibility arises at the posterior end of the embryo. This secondary region arises very early in *Fundulus,* later in the cod, and very late in the cunner.

7. After the origin of the secondary posterior region, the general susceptibility gradient in all three species is a "double" one. Anterior and posterior ends are the points of highest susceptibility and from them the susceptibility decreases in both directions towards the middle. Both ectodermal and mesodermal structures (somites) are involved in the double gradient but the ectodermal structures (neural tube) are in general much more susceptible.

8. The heart is highly susceptible (*Fundulus*). The venous end of the heart is the most susceptible part of it and from it the susceptibility decreases towards the arterial end.

9. Besides the general gradients, specific organs may exhibit high susceptibility. Conspicuous examples of this are the eyes (especially in *Fundulus*), the auditory vesicles, and the cerebellum.

10. The relations of these gradients to normal development are considered. It is pointed out that the embryo arises for the most part from material that does not come from the germ ring but that later the germ ring contributes to the embryo in degrees varying in different species. It is further pointed out that the germ ring type of development is probably a specialization from a method in which the center of the blastoderm played the chief rôle in development. The facts recorded do not support the theory of concrescence.

11. The oxygen consumption and carbon-dioxide production

of developing eggs of *Fundulus heteroclitus* increase up to the time when the germ ring is at the equator of the egg. Subsequently they decrease relative to the amount of protoplasm but show an absolute increase owing to the heart beat and other activity. This period when the respiratory metabolism is greatest is also the period when the eggs are most readily modified by external agents.

12. The relation of the susceptibility data to teratological development is discussed at considerable length. It is shown that those parts of the embryo having the highest susceptibility are those which are most defective in teleost terata and that such differential susceptibility is therefore the explanation of teratological development. It is also shown that these same parts most susceptible under extreme conditions may recover if conditions permit and may develop while the less susceptible parts fail to recover. Recovery forms of terata are thus just opposite in appearance to inhibited types.

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Explanation of Plates.

Plate I.

Figs. 1 and 2. Two stages in the disintegration of an early blastoderm of *Tautogolabrus,* showing greater susceptibility of the central cells.

Figs. 3 and 4. Two stages in the disintegration of an early blastoderm of *Fundulus,* showing rupture and disintegration of the central region.

Fig. 5. Disintegration of an early blastoderm of the cod, showing greater susceptibility of the margin.

Figs. 6 and 7. Disintegration of a later blastoderm of the cod, showing greater susceptibility of one region of the circumference and spread of disintegration in both directions from this region.

Fig. 8. A later blastoderm of *Tautogolabrus*; posterior half of the blastoderm most susceptible.

Figs. 9 to 11. Disintegration of a late blastoderm of *Tautogolabrus.* Fig. 9, the normal blastoderm; Fig. 10, bulging of the blastoderm up from the yolk and disintegration of the central posterior region; Fig. 11, further course of disintegration.

Fig. 12. First appearance of the germinal shield in the cod.

Figs 13 and 14. Disintegration of a stage like Fig. 12. Fig. 13, disintegration of the shield; Fig. 14, spread of the disintegration around the germ ring.

Fig. 15. First stage in the disintegration of a later stage of the embryonic shield of the cod; disintegration beginning at the anterior end of the shield.

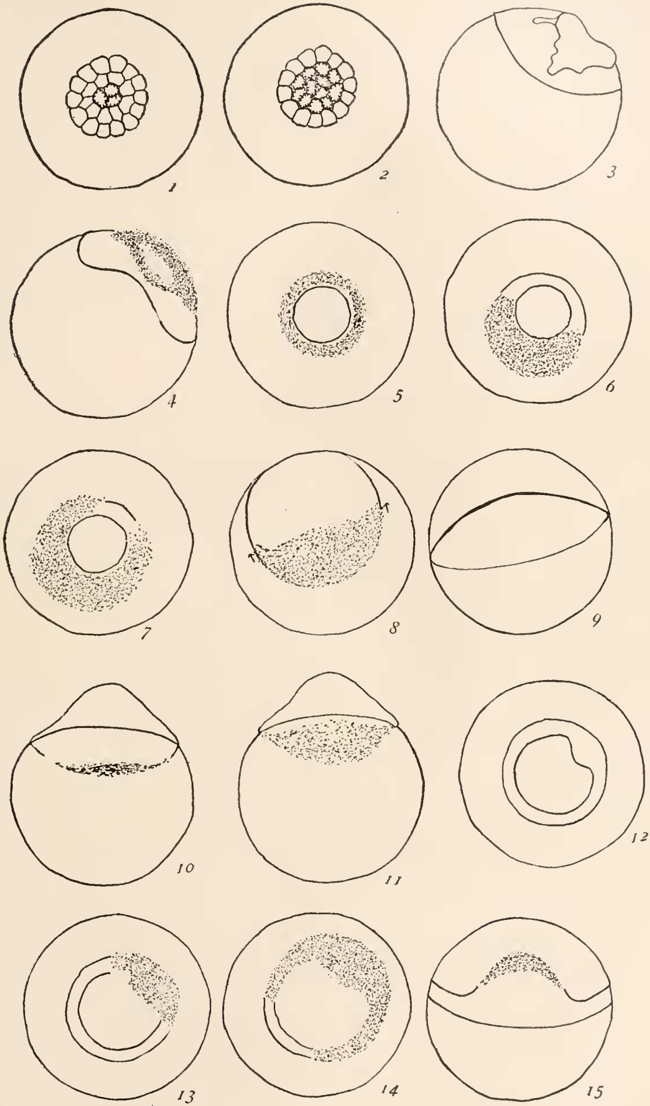

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Plate II.

Fig. 16. Further course of the disintegration shown in Fig. 15.

Fig. 17. Stage of the early embryonic axis in *Tautogolabrus*.

Fig. 18. Same blastoderm as in Fig. 17, drawn without the yolk; it is much shrunken. The embryonic region is disintegrating.

Fig. 19. Three stages in the disintegration of the earliest observed embryo of *Fundulus*. Anterior end to the left. Disintegration begins at the posterior end, then the anterior end, and proceeds in both directions to the middle.

Fig. 20. Earliest appearance of the embryo in the cod. Disintegration beginning at the anterior end of the embryonic shield.

Fig. 21. Further course of the disintegration shown in Fig. 20.

Fig. 22. Later embryo of the cod; disintegration beginning at the anterior end of the shield.

Figs. 23–26. Further course of the disintegration shown beginning in Fig. 22.

Fig. 27. An early embryo of *Tautogolabrus*. Disintegration is beginning at the anterior end of the head.

Fig. 28. Same embryo as Fig. 27, enlarged, showing course of the disintegration along the neural tube.

Fig. 29. An embryo of *Tautogolabrus* shortly before the closure of the germ ring.

Figs. 30–32. Three stages in the disintegration of the embryo shown in Fig. 29.

Fig. 33. Normal embryo of *Tautogolabrus* after the closure of the germ ring.

Fig. 34. Three stages in the disintegration of the embryo shown in Fig. 33. The somites are omitted in the first two drawings.

Fig. 35. An embryo of *Fundulus* after the appearance of the optic vesicles and three stages in its disintegration.

Fig. 36. A later embryo of *Fundulus* and four stages in its disintegration. The somites are omitted from the latter. The neural tube is characteristically curved.

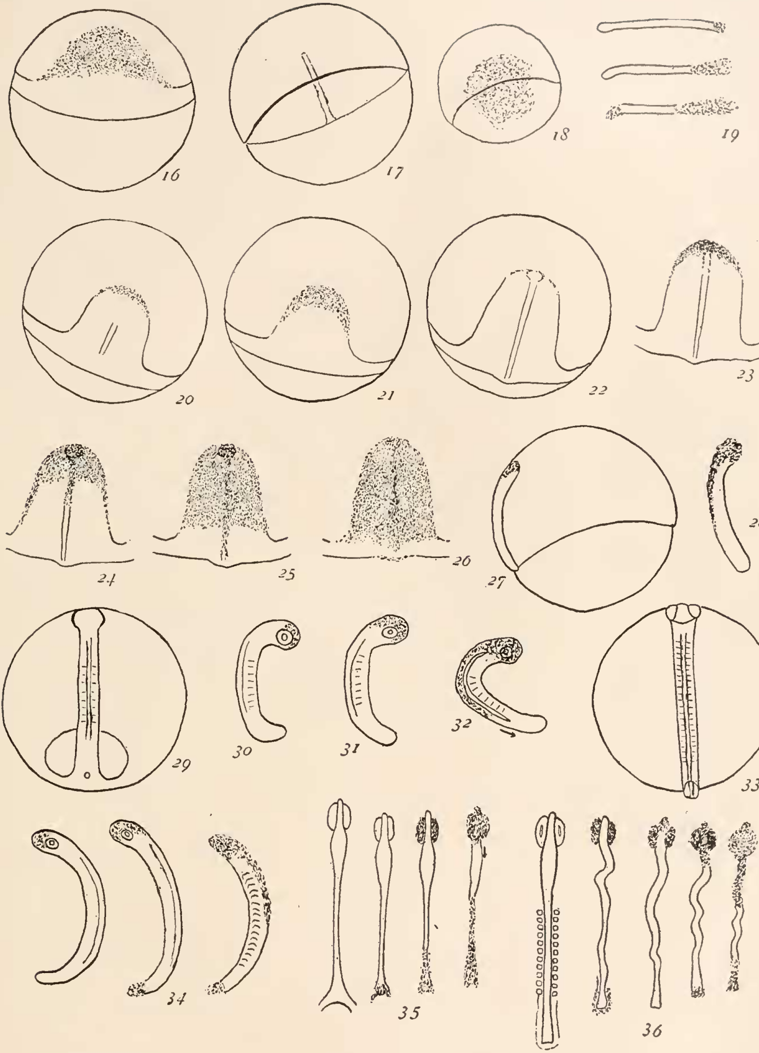

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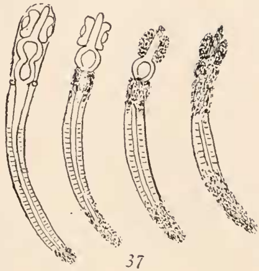
PLATE III.

FIG. 37. Four stages in the disintegration of a later embryo of *Fundulus*.

FIG. 38. Three stages in the disintegration of an embryo of the cod after the formation of the optic vesicles.

FIG. 39. Four stages in the disintegration of an embryo of the cod shortly before the closure of the germ ring.

FIG. 40. Four stages in the disintegration of an embryo of the cod after the closure of the germ ring.



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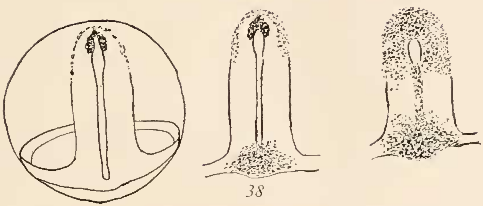

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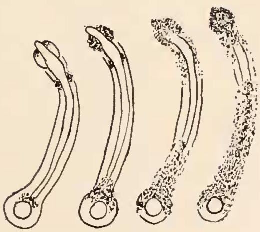

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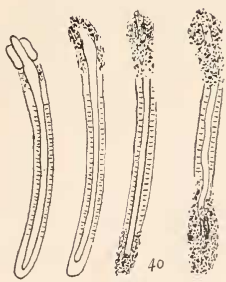

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OBSERVATIONS ON THE DISTRIBUTION AND HABITS OF THE BLIND TEXAN CAVE SALAMANDER, TYPHLOMOLGE RATHBUNI.

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When in 1895 the artesian well was drilled at the U. S. Fish Hatchery in San Marcos, Texas, the first specimens known to biologists of the blind cave salamander, *Typhlomolge rathbuni*, were brought up with the waters from the depths of the ground. The animals were described by Prof. L. Stejneger. For several years after this a relatively large number of the blind salamanders, about 100 a year, were found in the basin of the well, but gradually the number decreased and lately has been reduced to a few specimens a year.

When the question arose of subjecting this animal to certain experiments on metamorphosis, it became evident that a number of specimens sufficiently large for this purpose could be obtained only through an extensive search in the actual habitat of the *Typhlomolge*. With the aid of a special grant from the Rockefeller Institute for Medical Research an extensive study of the caves of San Marcos and environment was made by the writer and Mr. C. A. Campbell, at that time instructor in biology at Coronal Institute in San Marcos, during the months of August and September, 1916. So far as the number of animals obtained is concerned, the result was disappointing. But, on the other hand, several observations were made which seem to be of interest as regards the distribution and habits of the *Typhlomolge*

and which furnish valuable suggestions as to the methods which must be employed in order to procure a large number of animals. The writer hopes to stimulate a search for these salamanders on a large scale, in order to make this interesting form accessible to the experimental biologist who is in need of just such an animal as *Typhlomolge rathbuni* for attacking many important problems.

General Characters of the Region.

As pointed out, the specimens described first by Stejneger were found in the basin of the Artesian Well in the Fish Hatchery in San Marcos, and were carried up into this basin by the flowing water of the Artesian Well. During a two months' stay in San Marcos, we secured only two specimens from this basin, but five other specimens were found in three other localities, *i.e.,* in Frank Johnson's Well, in Ezell's Cave and in Beaver Cave.

In order to understand the conditions which might have led to the present distribution of *Typhlomolge* and because these conditions in the future may be an important guide in tracing the subterranean channels which the animals inhabit, a careful study was undertaken. It was found that the conditions in the three places where we found *Typhlomolge* are essentially similar to those existing in the locality from which the water of the San Marcos Artesian Well is derived.

Before describing the well and the caves in which we found *Typhlomolge* it is necessary to point out the geologic peculiarities of this area of Texas, since these conditions not only led to the formation of the caves but also to the present distribution of the *Typhlomolge.* Whether or not they are also responsible for the peculiar characteristics of the animal as Eigenmann and Stejneger assume, is an important question, the answer to which, however, cannot be given before extensive experiments on this species have been carried out.

San Marcos is located on the so-called Balcones scarp line. This line runs from Austin to Del Rio in a south-westerly direction and separates in a most distinct way the Edwards Plateau (north of the line) from the Rio Grande Plain (south of the line). It forms the escarpments of the plateau towards the plains. Along this line a faulting has taken place in Eocene time (Hill

and Vaughan, p. 260), during which the part that now forms the plain was thrown down and the northern part which now constitutes the plateau was left behind. In consequence of this faulting, any particular geologic stratum now lies deeper on the side thrown down than on the plateau.

It was apparently this faulting which has led to the formation of many cracks in the rock layers. The caves near the escarpments of the Edwards Plateau represent gigantic cracks. Besides this factor there is still another cause leading to the formation of caves in this region. The entire area of the Edwards Plateau constitutes a huge outcrop of the Cretaceous. In the soft strata of the various cretaceous formations of the plateau, numerous caves have been formed by the mechanical force of the water combined with its dissolving action. By this process most of the rivers of the Edwards Plateau have disappeared almost entirely from the surface, and their former beds are dry. These rivers have sunken beneath the surface where they flow in subterranean channels.

The San Marcos Artesian Well.

When the Artesian Well of the U. S. Fish Hatchery in San Marcos (Fig. 1) was drilled in 1895, a number of water reservoirs were opened up by the drill. At present only the water is used which rises from a depth of approximately 190 feet. Here a cave filled with water was opened up; in it the *Typhlomolge* lived. The water in this cave must have been under a pressure sufficiently high to carry it up 190 feet. The *Typhlomolge,* thus, lived most abundantly in water under high pressure and without any access to air except that present in the water. The water of this cave belongs to the so-called "sweet water" horizon of the Edwards limestone in which formation the cave is located.

We measured the temperature of the water as it comes out of the tube of the well as approximately 21.5° C. Among the fauna of the cave from which the water of the San Marcos Well rises, are particularly conspicuous two crustaceans, both unpigmented and eyeless, an isopod, *Cirolanides texensis* and a decapod, *Palæmonetes antrorum.* The latter species is of particular importance,

since so far it has been found to occur in all localities which are inhabited by the blind salamanders.

The cave of the San Marcos Well, thus, is characterized in the following manner: (1) It is situated in the Edwards limestone. (2) It contains water derived from the "sweet water" horizon.

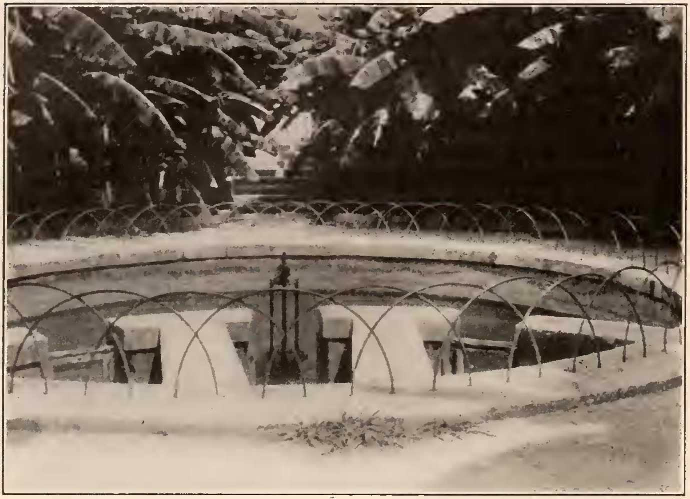

Fig. 1. Basin of the Artesian Well of the U. S. Fish Hatchery in San Marcos.

(3) The temperature of the water is approximately 21.5° C. (4) The water is inhabited by the decapod, *Palæmonetes antrorum*.

Frank Johnson's Well.

Approximately two miles southwest of the San Marcos court house (see map, Fig. 2), the flat valley of the dry Purgatory Creek crosses the Balcones scarp line opening here into the flat valley of the San Marcos River. Its northern slopes are formed here by the San Marcos Hill. Purgatory Creek originates near the Devil's Backbone, the divide between the Guadalupe and Blanco Rivers, at Boyett's Farm, about 14 miles northwest of San Marcos. It is dry at present, but several of the older in-

habitants claim that this creek had running water in it until about 50 years ago. At present only a few water holes are left in the upper course of the valley and several sink holes have formed in its lower course. These are filled temporarily with rain water. In time of severe cloud bursts the water in the creek becomes a

FIG. 2. Map of San Marcos Area.

1. San Marcos.
2. Artesian Well of U. S. Fish Hatchery.
3. Head of San Marcos River.
4. Beaver Cave.
5. Ezell's Cave.
6. Frank Johnson's House.
7. Frank Johnson's Well.
8. Swift's Cave.

torrent rising to a height of 8 feet, but it disappears completely within several hours.[1] Purgatory Creek has now become a subterranean creek. Mr. Frank Johnson's farm is located near where the creek crosses the fault line.

[1] The general character of a creek like this may be found described in Hill and Vaughan, page 207.

Mr. Johnson informed me soon after my arrival in San Marcos that the blind white salamander has been seen in his well, and in fact this well has yielded us more salamanders than any other place. It is shown in Fig. 3.

The well is located in the valley of Purgatory Creek, a short distance above where the creek enters the plain. Part of the flat valley is visible in the figure. Near the well is a sink hole (Driskel's Water Hole, see diagram, Fig. 4). The well was dug from

Fig. 3. Frank Johnson's Well. Showing the well house and at the right of the well house the dry and flat valley of the Purgatory Creek.

a level of 613 feet[1] above sea (San Marcos Court House 620 feet) to a depth of 31⅔ feet. There a cave was struck which now communicates with the well through a slit in the well bottom, as indicated in the diagram (Fig. 4). From this slit the water rose to from 3 to 5 feet in the well. This makes the sur-

[1] Altitudes above sea level were measured by means of an anaeroid barometer and therefore are only approximately correct (within several feet). Dimensions other than altitudes were measured directly, except when otherwise stated.

face altitude of the water about 584 feet. Mr. Johnson claims that the water is flowing. There is no doubt that Johnson's Well communicates with the subterranean Purgatory Creek. As in the case of the San Marcos Artesian Well, the water in this completely water filled cave must have been under a pressure sufficiently high to lift it to 3 feet in the well. It again is evident that the *Typhlomolge* prefer to live in water under high pressure and in caves which are filled entirely with water. The water of Johnson's Well has the same temperature as that of the San Marcos Artesian Well and also has the same taste. Besides the *Typhlomolge,* Frank Johnson's Well contains also the *Palæmonetes antrorum* and the *Cirolanides texensis.*

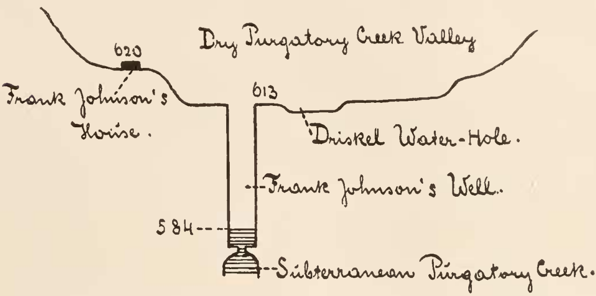

FIG. 4. Purgatory Creek Valley and Frank Johnson's Well. Diagrammatic section reconstructed from several cross sections. The figures indicate altitude above sea level in feet.

Thus, though the water of the Frank Johnson Well represents the subterranean Purgatory Creek, it shows great similarity to the water of the San Marcos Artesian Well. Particularly the presence in Purgatory Creek of 3 species typical of the artesian well would suggest that in some way the Purgatory Creek water is in communication with the so-called sweet water horizon near the San Marcos Artesian Well.

In Frank Johnson's Well the *Typhlomolge* were seen to pass through the slit from the cave into the well. During my stay in San Marcos, the water in the well was too high to catch the salamanders directly and for this reason traps were submerged in the well. These were ordinary minnow traps. In the beginning they

were supplied with various kinds of bait, but in this way only the crustaceans mentioned above were caught. The *Typhlomolge* did not seem to react to the bait, and later on when I observed the animals in the laboratory, it became evident that the instinct of hunger is not sufficiently strong in the *Typhlomolge* to make them go into traps; it is in fact very difficult to make these animals eat. Later on the traps were placed with one opening directly in the slit; animals passing out from the slit had to go directly into the trap. In this way 2 *Typhlomolge* were caught in Johnson's Well, one in August, 1916, and another in September, 1916. After I had left, 11 more *Typhlomolge* were found by Mr. C. A. Campbell and Mr. Rufus Smith who from time to time looked after my traps. Thus, Frank Johnson's Well yielded us 13 specimens of *Typhlomolge*. They were caught as shown in the following table. The number is, however, too small to warrant any conclusions as to a possible influence of the season upon the frequency of the occurrence of *Typhlomolge*.

August,	1916	1
September,	1916	1
November,	1916	2
December,	1916	1
January,	1917	2
April,	1917	1
Summer,	1917	2
November,	1917	3

One of the greatest difficulties encountered was to find a method of shipping the animals from San Marcos to New York; most of them did not survive the trip. In fact, only two ever reached the laboratory alive. The first seven specimens caught were taken on the train in a bucket filled with water. The jarring killed six. Among the eleven caught later on, only one survived the trip. Its safe transfer was accomplished by a fortunate incident. The animal was packed in a fruit preserving jar filled entirely with water and shipped in the winter. On arrival it was frozen tightly in a block of ice. This animal survived for one year in the laboratory. The only thing it could be made to eat were newly hatched larvæ of *Ambystoma maculatum*. Though kept for most of the time in a dark room, the skin which in the

beginning was white with a bluish, mother-of-pearl gleam, had darkened somewhat.

It should be pointed out here that slow reaction to food as exhibited by the *Typhlomolge*[1] is noteworthy in regard to certain findings of Miss E. T. Emmerson, who claims, upon anatomical reasons, a close relationship between *Typhlomolge* and the larvæ of *Eurycea rubra*. We are keeping a large number of such larvæ in the laboratory and contrary to my experience with the larvæ of *Ambystoma* and other salamander larvæ, these larvæ react very slowly to food. In fact, it is impossible to make them eat every day aside from the fact that most of the individuals of this species will eat only at night.

Ezell's Cave.

Ezell's Cave was opened up several years before the San Marcos Well was drilled. The entrance to the cave is located on the southwest slope of the San Marcos Hill (see map, Fig. 2), where it slopes down to the valley of Purgatory Creek about 2 miles W.S.W. of the San Marcos Court House, and not far from a little ravine, the bed of the dry City Boundary Creek, a tributary to Purgatory Creek. This location of Ezell's Cave indicates that it belongs to the Purgatory Creek System, the river found in it probably being the subterranean course of the City Boundary Creek.

Ezell's Cave distinctly exhibits the aspect of a large crack in the strata of the hill, brought about by dislocation of the strata towards the Purgatory Creek Valley. The entrance to the cave (approximately 670 feet above sea level) is part of a 62 ft. slit in the surface (Fig. 5), which for the most part is closed up by large rocks and runs from N.N.W. to S.S.E., that being the direction of the long axis of all the various parts of the cave. As the diagrammatic cross and longitudinal sections (Fig. 6 and 7) indicate, the entire slit so far as accessible is divided into two compartments by means of the rock masses which were thrown down during the process of dislocation and following corrosion.

[1] Normann, who kept a specimen of *Typhlomolge* in captivity, also reports great difficulty in making the animal eat.

These masses of debris form the bottom of the first story and in the N.N.W. corner leave open a small hole ("entrance hole"), $2\frac{1}{4}$ feet wide through which a narrow canal ("tube") may be reached which after running along the main axis of the slit for a short distance leads down into the second story or water room.

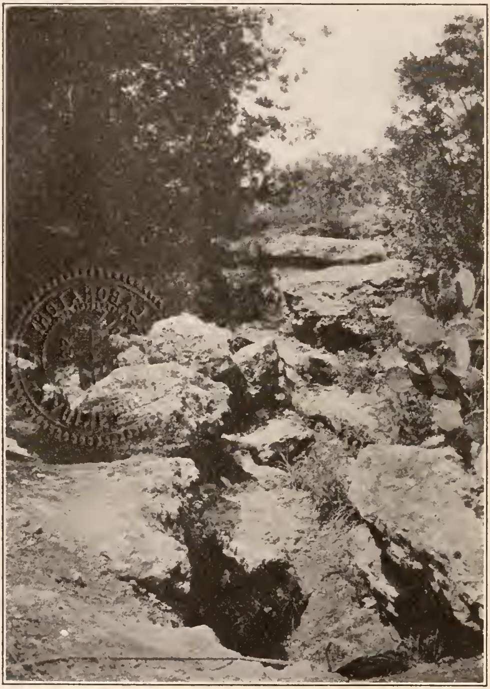

Fig. 5. Entrance to Ezell's Cave.

This compartment of the cave contains a large body of water (Fig. 8).

This pond is not formed by water which drains through the strata forming the roof of the cave nor by water flowing directly into the entrance of the cave, as the slope of the hill is drained in

the course of rain. The pond is formed by a subterranean river, which is evident from the fact that the water is flowing, though hardly in a perceptible manner. The flow can be observed from the dislocation of bodies dropped into the water at the N.N.W. end of the pond. If the water is not disturbed such bodies will arrive, in the course of an hour or so, at the S.S.E. end, thus indicating the direction of the flow. By means of a collapsible

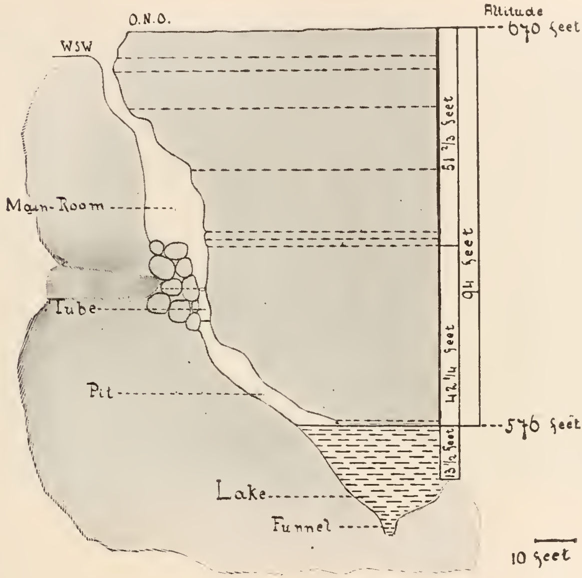

FIG. 6. Ezell's Cave. Diagrammatic section reconstructed from several cross-sections.

boat which was brought down into the water it is possible to follow the course of the subterranean creek towards N.N.W., (Fig. 9) for a distance of about 91⅓ feet. The crack extends, however, beyond this point and by climbing over a number of rocks the creek can be seen to continue in this crack. But we had no opportunity so far to explore this part of the cave.

The greatest depth of the water is 13½ feet, as far as it can

be measured. It is, however, not possible to ascertain exactly the depth of the water and of the crack, since the water is covered in part by the overlapping wall of the crack forming a ledge over the water (diagram Fig. 6 and photograph Fig. 9). Underneath this ledge the ground can be seen to slope down very deeply; it is possible by means of a strong light to see a funnel-

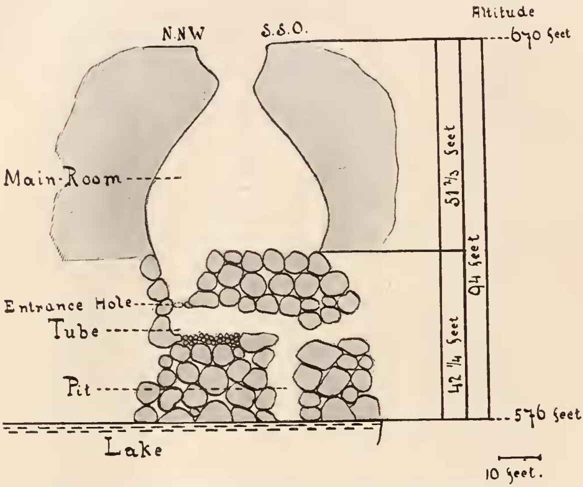

FIG. 7. Ezell's Cave. Diagrammatic section reconstructed from several longitudinal sections.

shaped crater opening at the deepest part of the lake in which no bottom can be seen.

The entire crack, with the water which it contains, is located in the Edwards limestone; but as pointed out above, the structure of the cave would indicate that this crack may extend into the deeper lying strata.

The distance from the entrance down to the water surface is 94 feet, which makes the level of the water about 577 feet. The altitude above sea level of the entrance of the cave was measured merely by means of a barometer, but the figure approaches the altitude of the water surface in Frank Johnson's Well near

enough; the water levels in Frank Johnson's Well and in Ezell's Cave are approximately equally high.

The water is of an extreme clearness and of bluish color, typical also of the water of the sweet water system. It also tastes like this water and has the same temperature (21.5°). Using a sufficiently strong light one discovers immediately a great number of *Palæmonetes antrorum* swimming near the sur-

Fig. 8. Water-room in Ezell's Cave.

face of the water, which thus contains also the same species of animals as were found in the water of the San Marcos Artesian Well and in Frank Johnson's Well.

Hence the water in Frank Johnson's Well and that in Ezell's Cave have a number of characteristics in common. They have the same taste, are of the same temperature, and their levels are equally high. They harbor the same species of animals. From their characteristics and from their location it seems that they are parts of the subterranean Purgatory Creek System.

Furthermore, both of these water bodies have certain most

conspicuous characteristics in common with the water of the San Marcos Artesian Well. They are of the same temperature and contain the same fauna. One naturally would think of a direct communication between the Purgatory Creek System and the caves which supply the San Marcos Artesian Well.

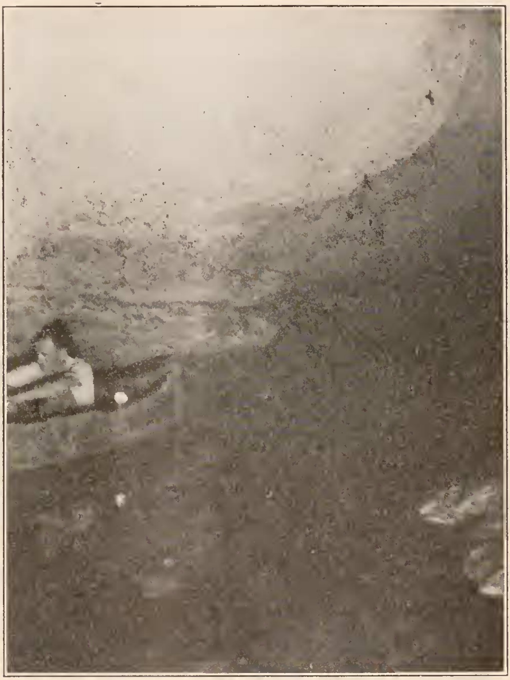

FIG. 9. Ezell's Cave Lake. Showing the overlapping ledge.

We caught only one animal (78.5 mm.) in Ezell's Cave. It was sitting quietly near the bank of the river where the water is shallow, and did not seem to mind pebbles dropped down into the water near it, nor the glare of the light from two Columbia dry cells. We spent 12 days in the cave under the most varied con-

ditions, and conducted a most extensive search for *Typhlomolge*. Hence the scarcity of this species is somewhat perplexing. It is possible that the animals prefer to stay further down in the passages and cracks filled completely with water under high pressure, an assumption which is supported by the circumstances under which the animals were found in both the artesian well and Frank Johnson's Well. It may be that they rarely and only by some incidental circumstances are induced to come to the more open bodies of water.

So far as is known to the writer, the specimen of *Typhlomolge* caught in Ezell's Cave in 1916 is the first and only one positively known as having come from this locality. But it is claimed by people in San Marcos, as Mr. S. N. Stanfield, teacher of biology in the Texas Normal School in San Marcos, informed me, that the first two *Typhlomolge* ever seen were found in Ezell's Cave, 1½ years before the well was drilled, in a small boat which had sunk in Ezell's Cave Lake.

Beaver Cave.

Not far from the entrance of Ezell's Cave on the southwest slope of San Marcos Hill and at an altitude of 652 feet above sea level, near the dry bed of the City Boundary Creek is situated the entrance to Beaver or Wonder Cave. The location of the cave would indicate that it belongs, like Ezell's Cave, to the Purgatory Creek System.

Beaver Cave represents the aspect of a straight running crack in the strata of the Edwards limestone, the same as Ezell's Cave; this crack, in part, has been widened out and its walls have been smoothed down by the action of the water (Fig. 10). Its bottom is made up of huge masses of broken-down rocks which form, at some places, high cliffs and rock masses, dividing the entire cave horizontally in a number of rooms connected by narrower tubes with one another, and vertically into several compartments. Fig. 11 represents a diagrammatic longitudinal section through the cave, which gives an idea of the construction of this cave. In Fig. 10, which was taken parallel to the longitudinal axis, the slit-like shape of the cave is shown; it can also be seen how smooth

the walls have been washed by the water entering easily through the thin roof of the cave.

The longitudinal axis of Beaver Cave runs from N.N.E. to S.S.W., forming an angle of approximately 25° with the longi-

FIG. 10. Interior of Beaver Cave. Photograph taken from rock 34 towards board rock. In back of the right hand side wall at its lower end, the opening of the "tube" is visible.

tudinal axis of Ezell's Cave; the length of the entire slit is nearly 500 feet. It is claimed that there is a direct connection between Beaver Cave and Ezell's Cave. We could not verify this statement, and it seems certain no one has actually found a connection. We found that at x in room VI. (see Fig. 11) a number of

tightly packed rocks and masses of gravel make further penetration impossible at present and that the location of both caves and the direction of their main axes are not in favor of the statement mentioned above.

The deepest depression in the bottom of Beaver Cave is found in the room indicated in the diagram Fig. 11 as "Well-Room." The bottom of this floor is 62 feet below the surface and therefore at a level of 590 feet above sea level. As seen from the height of the water level in Johnson's Well and in Ezell's Cave, no water of the Purgatory Creek System should be present in Beaver Cave. And in fact when the cave was discovered there was no water found. But a well drilling made at that time from the surface above the Well Room had indicated the presence of water only a few feet beneath the bottom of the Well Room. Therefore, a hole was dug in the bottom of the Well Room which led to water at a depth of about 3 feet or at the same level as the surface of the water in Ezell's Cave and Frank Johnson's Well (see Fig. 13).

At present one finds in the Well Room of Beaver Cave a rectangular basin approximately 6 feeet in length, 3 feet in width and 6 feet in depth, the bottom of which is covered with mud and rocks, and the walls of which are lined with logs.

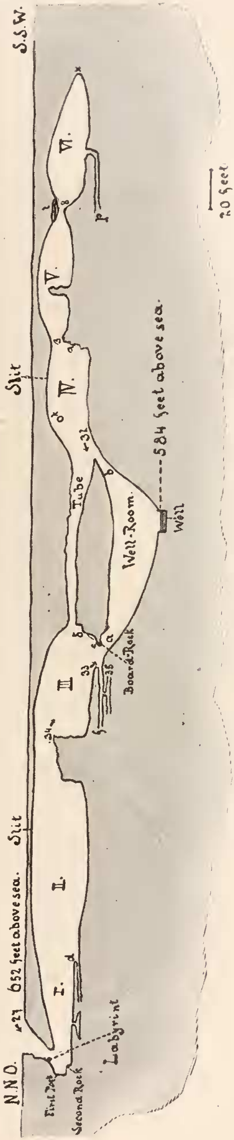

FIG. 11. Beaver Cave. Diagrammatic section reconstructed from several longitudinal sections.

This basin is filled with water half of its depth. Hence the surface of the water stands at the same level with the surface of the water in Frank Johnson's Well, and the suggestion seems justified that in this basin again part of the Purgatory Creek System was opened up. The water has the same taste as the water of the other localities mentioned and also has the same temperature (21.5° C.). In which way, however, this basin in Beaver Cave could be connected with the other localities cannot be stated with certainty at present, since the log lining of the wall made it impossible to search more closely whether or not the rocks of the wall contain any larger cracks or crevices. It also was not determined whether the water is flowing. But its clearness and the fact that the mud when stirred up disappears in a relatively short time would speak in favor of a slight current in the water. There is, however, one fact which hardly could be explained in any other way than that the water in the basin must be in connection at least at certain times with some larger bodies of water. The well in Beaver Cave contains both the *Palæmonetes antrorum* and the *Cirolanides texensis,* animals the transmission of which to the basin since it was constructed must have taken place by means of water currents which drive water from certain water bodies (harboring these animals) through the well.

Hence it is most probable that the water of Frank Johnson's Well, of Ezell's Cave and of Beaver Cave is the water of the subterranean Purgatory Creek System.

In the well of Beaver Cave two *Typhlomolge* were caught, one by means of a dip-net, the other in a trap which was laid with its opening just in front of a hole into which the animal had been seen to pass. One specimen was 82 mm. in length, the other one the largest caught measured 120 mm. Both these animals were observed for some time before they were actually caught; they proceeded to move in characteristic fashion—as described very accurately by Normann—by intermittent walking and resting in the presence of light. Even when the rays fell directly upon them, they did not seem to be disturbed. In this respect our observations made in the animal's natural habitats, agree very well with the observations made by Normann in the labora-

tory. Pebbles and a pocket-knife dropped into the water near the animal did not change its behavior; we have not found that the *Typhlomolge* as Normann claims possesses a specially high sensitivity towards disturbances of the water. Once stirred up the animals immediately swim towards the walls, and if they cannot find cover immediately, they swim along the wall toward the surface pushing out their snouts above the surface.

Before I was acquainted well enough with the general situation in the localities in question and before I had other facts indicating a possible connection between Beaver Cave and the Purgatory Creek, the occurrence of the *Typhlomolge* in the Beaver Cave well was puzzling, since it seemed to be difficult to explain how they could have been transferred to the well. In an anatomical study performed on *Typhlomolge rathbuni,* E. T. Emmerson points out the close relationship existing between *Typhlomolge* and *Eurycea* (*Spelerpes*), in particular *Eurycea rubra* and suggests that *Typhlomolge* may be the larva of an unknown species of the genus *Eurycea.* The writer of this article has a large number of larvæ of *Eurycea rubra* under observation and finds that in certain habits (feeding and especially the pushing out of the snout above the water when aroused) a remarkable resemblance exists between *Typhlomolge* and *Eurycea rubra,* a resemblance which was not observed by the writer in larvæ of the many other species of salamander closely watched in the laboratory. Concerning, however, the assumption that *Typhlomolge* is the larva of some species of *Eurycea,* this meets with one difficulty if it should mean that this species is still in existence. Ezell's Cave and especially Beaver Cave were closely searched for the presence of other salamanders. None were found in Ezell's Cave. In Beaver Cave, however, Mr. Campbell found about 20 specimens all belonging to the species *Plethodon glutinosus;* this is the only salamander which we could detect in these and other caves of the area around San Marcos. In view of this fact it appears that the suggestion as to whether or not *Typhlomolge* is the larva of a species represented at the present time also by metamorphosed specimens would be hardly more than speculation. It is, however, certain that it would be of the

greatest value to raise the *Typhlomolge,* in order to study closely their mode of propagation, development and to subject these animals to certain experiments indicated by our present technic in the study of the metamorphosis of other salamanders.

In connection with the metamorphosis of *Typhlomolge* it may be pointed out that Miss Emmerson has made a statement which is so important that it arouses curiosity as to why it has attracted so little attention. Miss Emmerson searching for the organs of internal secretion of *Typhlomolge* found that the animal possesses a thymus gland, but she could not find a thyroid gland. If the lack of a thyroid gland could be confirmed—and we are preparing some of our specimens for examination with that end in view—Miss Emmerson's discovery will explain why the *Typhlomolge* cannot metamorphose at present, since Allen has demonstrated that larvæ of frogs and toads whose thyroids were extirpated did not metamorphose, though the controls with intact thyroids all metamorphosed. Do the Proteidæ (*Proteus*) possess thyroids, is the lack of the gland common to all of them? And what are the reasons for the atrophy of the gland? These are problems which call urgently for investigation.

From the facts mentioned above it is certain that the *Typhlomolge* inhabit the subterranean waters which constitute the Purgatory Creek System and a subterranean water channel which supplies the San Marcos Artesian Well. These two systems are located north and south respectively from the Balcones scarp line. On account of the faulting, though both the Purgatory Creek Caves and the Artesian Well Cave are located in the same geological formation, the latter cave occupies a position several hundred feet deeper than the Purgatory Creek Cave; this is indicated in the diagram, Fig. 12. The water in both systems is of different origin, as may be seen from this diagram. The water of the Artesian Well is the so-called "sweet water," which on the plateau, *i.e.,* in the region of the Purgatory Creek System, is carried in beds below those in which the caves of the Purgatory Creek System are located. The "sweet water" is caught by the basement beds of the Cretaceous, the Travis Peak and Glen Rose formation, where they outcrop on the plateau, and is carried

down along the slanting stratum beneath the geologically higher situated Edwards limestone and towards the fault. Along the fault, however, the continuity of the water-bearing strata is broken and they come to lie in one level with the Edwards limestone of the plain; thus, here the water is forced from the Glen Rose formation into the Edwards limestone.[1]

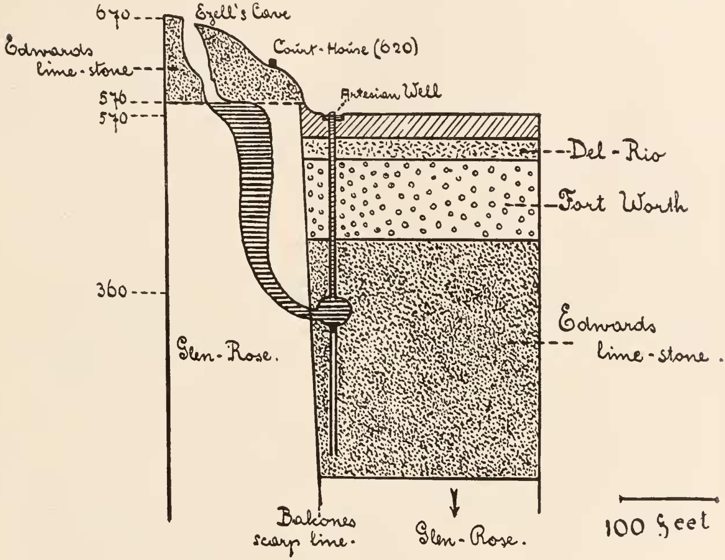

FIG. 12. Ezell's Cave. Balcones scarp line and Artesian Well of U. S. Fish Hatchery in San Marcos, Texas. Diagrammatic section showing the position of northern and southern part of the various cretaceous formations to each other after the dislocation of the Rio Grande Plain in Eocene time accomplished by faulting. The figures on the left-hand side of the diagram indicate altitude above sea level in feet.

The water of Johnson's Well, Ezell's Cave and Beaver Cave, however, is the river water of the subterranean Purgatory Creek. The level of the Purgatory Creek in these localities at present is at an altitude of about 580 feet, that of the Artesian Well 360 feet.

[1] Hill and Vaughan, page 315.

But as indicated above, it is quite probable that a direct communication has been established between these two systems by means of channels which according to our calculations would have a depth of about 200 feet; if this is a fact, the relation between the various bodies of water in question would be as shown in the diagram of Fig. 13.

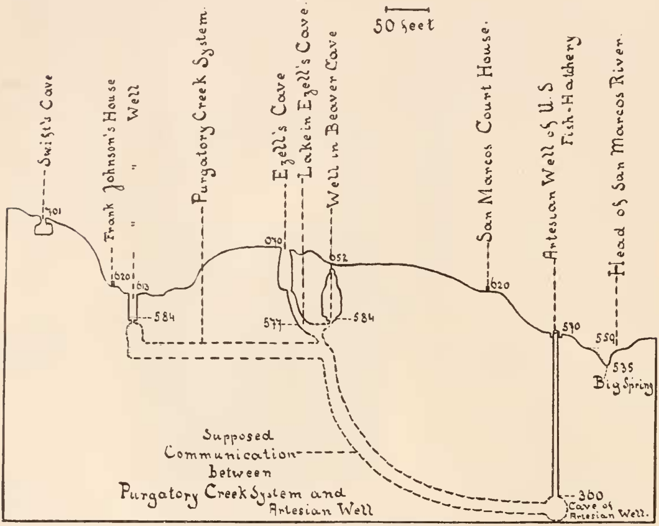

Fig. 13. Purgatory Creek System. Artesian Well and San Marcos Springs at San Marcos, Texas. Diagrammatic section reconstructed from several sections, showing the way in which these waters probably communicate with each other. The figures indicate altitude above sea in feet.

It is of great importance to ascertain whether or not such a communication exists, since this would facilitate following the *Typhlomolge* along the course of travel and since it would permit conclusions as to the mode of the distribution of the species. Besides the suggestive structure of Ezell's Cave there are a number of facts which are in favor of the existence of a communication. If no connection between the two systems exists it would mean that the *Typhlomolge* lived in the subterranean rivers be-

fore the present southern and northern parts of the Edwards limestone were separated from each other, and that after the dislocation in Eocene time part of the species was caught in the caves of the Edwards limestone of the San Marcos area south of the Balcones where it lived completely isolated from the rest of the species. Since the specimens obtained from Ezell's Cave and the Artesian Well are identical, it would mean either that the species remained absolutely unchanged since Eocene time, or if it changed, underwent exactly similar changes in the open ponds of the subterranean Purgatory Creek and in the completely closed and water-filled subterranean caves of the Artesian Well. It is evident that none of these possibilities is probable.

Not only the Artesian Well at San Marcos but numerous other artesian wells along the Balcones escarpments are supplied from the sweet water horizon; yet from none of them, except the San Marcos Well, *Typhlomolge* has ever been reported. This would be explained if the San Marcos Well contains besides the sweet water also the Purgatory Creek water, since this certainly could not be true for the other wells. Probably the Purgatory Creek is the original habitat of the *Typhlomolge* and later on the animals migrated down to the water channels of the Artesian Well.

Also in none of the fissure springs of the Balcones scarp line, not even in the San Marcos springs though they all come from the sweet water reservoirs, *Typhlomolge* ever has been collected. The same explanation as to the artesian wells could be applied to these springs, if a communication exists between the Purgatory Creek System and San Marcos well.

Finally an incident may be mentioned here which also would speak in favor of the existence of a direct communication between the Artesian Well and the Purgatory Creek System. Mr. Mark Riley, superintendent of the U. S. Fish Hatchery, informed me that in the basin of the Artesian Well a number of catfish were kept at one time, but they disappeared gradually from the basin and it is claimed that they migrated into the tube of the artesian well. The writer is not prepared to form an opinion concerning the probability of such migration. One day, however, while I was looking for *Typhlomolge* in Ezell's Cave, I saw some fishes

hiding behind the rocks. Shortly after this we caught two fishes by means of hooks which were placed near the rocks where I had seen the fishes; both were catfish. And they were the only specimens of fish which I ever saw in Ezell's Cave during the 12 days I spent there. If these were identical with the individuals kept in the basin of the Artesian Well, it certainly would be proof of the existence of a communication between the Purgatory Creek System and the San Marcos Artesian Well. It would be of great importance to trace the course of the water in Ezell's Cave and Johnson's Well down to the reservoir of the Artesian Well. As suggested by the possible migration of the catfish, such methods could be easily designed and will be employed as soon as the investigations can be continued.

In case of a connection between the two systems, the water contained in each one would be a mixture of the Purgatory Creek water and the sweet water. In all four places in question the water has the same taste and the same temperature. It contains besides the *Typhlomolge* a number of typical species, among them the *Palæmonetes antrorum,* which I found to occur in all four localities.

Other Localities in Purgatory Creek Valley.

After it was found that the *Typhlomolge* inhabit subterranean regions probably representing the Purgatory Creek System, it was interesting to visit other caves of the Purgatory Creek. One of them is Swift's Cave, on the slope closing the valley towards southwest and abuot 1 mile above Frank Johnson's Well. The entrance to the cave is situated at an altitude of 701 feet above sea level. Though no water could be reached so far—according to what has been said above, it would have to be found about 114 feet below the entrance—there is in this cave a narrow tube leading down which has not been followed; further examination may reveal the presence of some passages to the water.

Further up the valley 14 miles above San Marcos, Boyett's Cave is located at an altitude of about 1,100 feet; there the Purgatory Creek valley starts. No water was found in Boyett's Cave down to a depth of 50 feet and no passages leading further

down were discovered. But in the large main hall of this cave one notices along the walls a whitish deposit for about 3½ feet above the ground and forming a straight horizontal line running along the walls, as seen in Fig. 14. This indicates the former presence of water in this cave. Probably with the general dis-

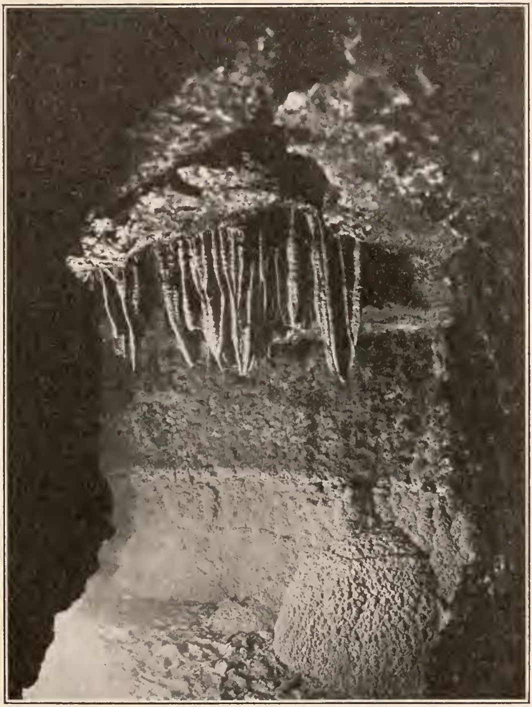

Fig. 14. Boyett's Cave.

appearance of the water from the Purgatory Creek valley and its fall to deeper levels, flowing water has disappeared from the cave. There are, however, a number of small shallow pools (several inches deep) formed from dripping water in the sandy bottom

of the main hall. In these pools certain crustaceans are found in large numbers, which according to Dr. Ortmann, are at least very closely related to if not identical with the species *Stygonectes flagellatus,*[1] an amphipod known from the San Marcos Well. Thus, this animal, which through its mode of living is well adapted to the conditions prevailing at present in Boyett's Cave, is the only remnant there of the Purgatory Creek System fauna.

The San Marcos Springs.

According to Hill and Vaughan it is quite certain that the San Marcos Springs, like all other fissure springs along the escarpments of the Edwards Plateau, are of the same origin as the artesian wells of this area, and hence the water of the San Marcos Springs comes from the same reservoir which supplies the Artesian Well at San Marcos Fish Hatchery. We might expect therefore that between these two localities ways of communication exist along which the *Typhlomolge* may travel.

We have not so far subjected the San Marcos Springs to a thorough examination, but a brief mention may be made of certain facts valuable for future exploration. The water of the San Marcos Springs comes from funnel-like depressions of the surface (see diagram, Fig. 11), and forms a little lake which is the head of the San Marcos River. The openings through which the water emerges lie deeper (at an altitude of only 532 feet) than the surface of the water of Purgatory Creek. The surface of the lake which is artificially dammed, is 559 feet above sea level. The temperature of the water in a little spring on the bank of the lake is 21.5° C., like that of the water of the Purgatory Creek system and the Artesian Well.

Towards the south the valley at the head of the River continues and forms the bed of the San Marcos River in which the water is flowing, but this valley can be traced also north of the springs, though here it is dry.

Unconfirmed claims have been made that the "white salamander" was seen at the head of the river, but that it had developed eyes and turned brownish. These statements are, no

1 For further information see Benedict and Weckel.

doubt, due to the occurrence there of the larvæ of other salamanders which have been mistaken by the layman for *Typhlomolge*. But even if the animal should come up into the lake, it would be quite difficult to find it there; unaccustomed to such rapacious enemies as certain fishes which abound in the lake, the blind *Typhlomolge* would soon fall a victim.

There are, however, two localities further up in the dry valley which it might be important to examine. One is a hole resembling a well hole because of its regularity. It is about 6 feet deep. Mr. Bidler who is well acquainted with conditions as they were 20 to 30 years ago in this area, informed the writer that at one time this hole was much deeper and contained a small body of water. He assured me that one day in the water of the hole two white *Typhlomolge* were seen. At any rate this hole should be prepared for examination by removing the gravel and rocks and thus penetrating to the water, the level of which would be approximately that of the water in Purgatory Creek. The place could be easily prepared so as to make trapping there a success.

Near this place on one of the slopes of the valley is situated another hole (on the property of Mr. Mark Riley), which much resembles the entrance to Beaver Cave. In former years, before the water which abundantly drains into that hole had washed gravel into it, one could penertate it for some distance and reach a place at which the sound of water could be heard.

The writer believes that the exploration of the two places mentioned would lead to a definite knowledge about the presence of *Typhlomolge* in the San Marcos River valley.

Other Places to be Explored.

It is clear that *Typhlomolge* cannot be procured in abundant number by collecting in one or two places only, since these animals pass only in small numbers from the deep water-filled caves into the open water bodies of the higher horizons. Successful collecting must have as a basis the discovery of a large number of places where some of these animals can be found. Traps must be laid in all these places and watched for some time. Assuming that from three such localities 5 specimens could be obtained in

the course of two months, as was the case in Ezell's Cave, Beaver Cave and Johnson's Well, a steady and skillful worker could collect from 18 such places 180 specimens in a year, a number sufficiently large to start experimental work on the species and to keep a sufficient number for breeding stock. For this reason an attempt will be made to mention briefly a number of other places where *Typhlomolge* may possibly be found.

There are two caves on the ranch of Mr. Bender at Spring Branch, 40 miles above San Marcos and about 1,100 feet above sea level. One is a narrow channel through which the head water of Spring Branch Creek passes out. The channel is filled almost to the top with water but it is possible to penetrate it to a depth of 350 feet. Since the water is flowing quite rapidly, it is not likely to contain *Typhlomolge,* but a more thorough search might be conducted. The other cave represents a narrow crack in the strata containing water at a depth of 45 feet. It is only a small pool, which, however, is part of a larger body of water covered by overlapping ledges. The temperature of the water is 20.5° C. Besides frogs, some other animals inhabit this pool. They could not, however, be identified, as upon our approach they immediately dived underneath the ledge.

More important still is the water on Mr. Bremer's ranch, at the water hole of the Cypress Fork in Hays County, a tributary branch of the Blanco River, about 1,000 feet above sea level. The water hole (Jacob's well) itself is filled with blue water which has a temperature of 22.5° C. On account of the large black basses inhabiting the hole one would not expect to find *Typhlomolge* there. But further up on one of the slopes of the dry valley is located the entrance to a cave in which the water (probably of Jacob's well) could be reached. We penetrated to a place where a number of small holes perforate the bottom of the cave; pebbles thrown into the holes evoked the sound of rather deep water. By dislocating a large rock, it would be possible to make one of the holes large enough to gain access to the water.

It might be valuable to mention a few places in which, according to Mr. S. A. Stanfield, *Typhlomolge* have been seen:

Burnet Cave, Kendall County, near Burnet.

A spring near Twin Sister Mountain, Hays County, 2 miles from Wimberly.

A spring near Ozona, 100 miles from San Marcos.

Summary.

1. At present it is certain that *Typhlomolge rathbuni* inhabits the subterranean water of the Purgatory Creek System just north of the Balcones scarp line and one mile further up, and the caves of the Artesian Well of the U. S. Fish Hatchery at San Marcos, which seem to be in direct communication with the Purgatory Creek System by means of channels about 200 feet deep.

2. The populations of the species *Typhlomolge rathbuni* north and south of the present Balcones scarp line have not been separated from each other by the process of faulting in Eocene time, but have developed in unrestricted communication with one another.

3. No certain data are available as regards the occurrence of *Typhlomolge* in the San Marcos Springs and in the dry valley of the San Marcos River north of the Springs. Since the Springs come from the same water reservoir as the Artesian Well, further investigations should be conducted.

4. All the localities containing *Typhlomolge* are located in the Edwards limestone region, but the caves of the Artesian Well are 200 feet deeper than the rest.

5. *Typhlomolge* have been found in the Purgatory Creek System at an elevation of approximately 585 feet above sea level. Where this level could not be reached as in the upper Purgatory Creek valley, only remnants of the Purgatory Creek System fauna (*Stygonectes flagellatum*) were found.

6. The water inhabited by *Typhlomolge* seems to be slowly flowing water.

7. The temperature of the water is approximately 21.5° C. and it is inhabited by the decapod *Palæmonetes antrorum.* Since the latter animal is much more numerous and can be detected much easier than the *Typhlomolge,* its presence may be taken as an indication that the place is promising as regards the presence of *Typhlomolge.*

8. The rarity of the *Typhlomolge* seems to be due to the animal's habit of preferring deep lying cracks or crevices, completely filled with water at a higher pressure than exists in the more open bodies of water located at higher levels.

9. As regards the habits of the *Typhlomolge* in its natural habitat we were able to confirm Normann's observations made in the laboratory in respect to the peculiar mode of walking of this animal and its indifferent attitude to light. But we did not find the animal particularly sensitive to water waves.

10. In feeding and swimming when aroused, *Typhlomolge* shows a close resemblance to larvæ of *Eurycea rubra.*

11. The assumption, however, that *Typhlomolge* is the larva of some unknown and still existing species of the genus *Eurycea* as made by Emmerson could not be confirmed, since with the exception of the species *Plethodon glutinosus* no tailed Batrachians were found in the caves. More important than this assumption is the fact that *Typhlomolge,* according to Emmerson, lack a thyroid, which would explain why these animals cannot metamorphose.

12. In order to collect a large number of specimens necessary for experimental work and intensive study of the species, as many places as possible must be discovered which may contain *Typhlomolge,* and collecting must be conducted simultaneously in all these places.

13. The best method of catching the animals is by trapping, but this method must be improved. It seems probable that live bait is not attractive to the animals. Instead of relying upon bait, the large openings of the traps should be laid in the path of the animals.

I desire to express my indebtedness and warm thanks for the assistance which they have so generously rendered me in this work, to the persons whose names I take pleasure in stating below:

Mr. C. A. Campbell, instructor in biology at Coronal Institute in San Marcos, for his enthusiastic and skillful assistance and his most enjoyable company during the collecting trips.

Dr. H. F. Moore, Acting Commissioner of U. S. Fish Hatch-

eries, for permission to keep apparatus and outfit as well as material collected at the U. S. Fish Hatchery in San Marcos.

Mr. Frank Johnson for permission to use and abuse his well in every conceivable way and for valuable suggestions and help in catching *Typhlomolge* out of the well.

Mr. Mark Riley, Superintendent of U. S. Fish Hatchery in San Marcos for aiding the work in every possible way.

Dr. W. T. Vaughan, of the U. S. Geol. Survey, and Prof. C. Eigenmann for valuable suggestions as to traveling and local conditions in Texas.

Dr. T. W. Stanton and Mr. L. W. Stephenson, of the U. S. Geol. Survey, for determination of the various rock specimens collected from the caves.

Dr. H. E. Ortmann for identification of the various species of Crustaceans.

Dr. L. Stejneger for identification of the salamander *Plethodon glutinosus*.

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BY THE BIBLIOGRAPHIC SERVICE, FEBRUARY 7

ON THE DEVELOPMENT OF THE SPONTANEOUSLY PARTHENOGENETIC EGGS OF ASTERINA (PATIRIA) MINIATA.

H. H. NEWMAN.[1]

Introduction.

While engaged in a series of experiments on echinoderm hybridology, which I was conducting during the months of April and May, 1920, at Pacific Grove, California, I was forcibly struck by the frequency with which spontaneous parthenogenesis occurs in the starfish, *Asterina* (*Patiria*) *minata.* When I use the term "spontaneous," I mean that eggs were in no way treated either by physical or by chemical agents. Precautions were taken, moreover, to prevent accidental fertilization. The procedure was as follows:

Sea water, brought in from the open sea and therefore free from the chemical impurities present in sea water that has been pumped through metal pipes, was allowed to stand at least four days at laboratory temperatures, which during the month of May ranged from 16° to 19° C. It is certain that no sperms could live for this length of time in sea water. This method is chosen in preference to Loeb's practise of heating the sea water to 60° C. for some time, because it involves no possible chemical changes in the sea water nor any driving out of oxygen. Before opening a starfish, it was scrubbed thoroughly in cold running fresh water and rinsed in a strong stream of fresh water. In case the animal proved to be a male it was discarded, and hands and instruments were scrubbed in fresh water before touching another starfish. If the animal proved to be a ripe female the ovaries were gently shaken into a finger-bowl containing 150 c.c. of the sea water prepared for the purpose and the bowl was covered with a clean glass plate and placed upon a table out of reach

[1] From the Hopkins Marine Station of Leland Stanford, Jr. University and the Hull Zoölogical Laboratory of the University of Chicago.

of direct sunlight. For purposes of observation eggs were removed from time to time and placed in watch-glasses with a sterilized pipette. It was thought safer not to run, as direct controls, normally fertilized eggs, but on other days and under identical conditions numerous observations were made on the course of normal fertilization and development. The differences observed between the behavior of parthenogenetic and that of fertilized eggs were so striking that it seemed well worth while to study and to describe them.

Peculiarities of the Material Used.

Asterina (*Patiria*) *miniata* is one of the commonest starfishes of the California coast. It is a relatively small species in which the five rays are almost completely amalgamated with the central disc in such a way as to give the creature a nearly pentagonal outline. In color it ranges from a brilliant scarlet to a light cream color with various intergrades and piebald combinations. It seems likely that there are several subspecies that freely interbreed. My experience seems to indicate that relatively few eggs mature at a time and are exuded in small numbers over a long season. Among the very large number of females examined I never found an ovary that showed any large percentage of ripe eggs. Those that were shed from the removed ovary and gently shaken in sea water contained oöcytes in all stages of development, some quite small, others fully grown but incapable of maturation, still others mature and ready for maturation and fertilization after standing about one and a half to two hours in sea water. None of the eggs when freshly shed had undergone maturation. This may mean that the artificial shedding of the eggs is a premature process, and that, as seems to be the case in some asteroids, if the eggs were to be normally extruded through the genital pores, they would be immediately ready for fertilization. I have never been able to observe the extrusion of eggs in *Asterina,* nor to induce it by massaging, as can be done in some asteroids. This may possibly be due to some peculiar sexual rhythm in this species, which results in ovulation occurring only at night or at some particular phase of the moon or of the tide.

On this point I have no evidence. So it should be borne in mind that the eggs used in these experiments, and presumably by Loeb, who did a considerable amount of work upon artificial parthenogenesis in this species, are not the normally shed eggs equivalent to those which are fertilized in nature, but are probably prematurely shed eggs. It is very questionable, therefore, whether parthenogenesis ever occurs in nature. It seems more probable that the parthenogenetic eggs observed in these experiments result from the artificial conditions involved in shedding some of the eggs prematurely.

The eggs of *Asterina* are very hardy and resistant of the cytolytic action of sea water. While the unmaturated oöcytes of most echinoderms begin to disintegrate within twenty-four hours, those of *Asterina* frequently remain unchanged, as though in stable equilibrium, for from four to eight days. I have before me a number of microscopic whole mounts, showing numbers of eggs and larvæ of *Asterina* fixed in Bouin's solution on the eighth day after fertilization, in which there occur a number of unmatured oöcytes with germinal vesicle clean-cut and spherical and plasmosome well defined. This ability of the unripe eggs to withstand disintegration is of great practical value in the study of development, for the water is kept free from the products of egg decay; a decided advantage in view of the fact that where there are small percentages of developing eggs surrounded by large percentages of non-developing eggs, the former would have very small chance of survival if the latter were to decay and foul the water.

Experimental Data.

Most of the detailed data on pathenogenesis in *Asterina* were obtained during the month of May, 1920, although the phenomenon was noted incidentally throughout April. The month of May seems to be the best month for work with *Asterina* as there appear to be larger ovaries and more full-grown oöcytes then than earlier. Possibly June would be still better, though I have not tried any experiments at that time.

Some thirty-two experiments were made in all, and no two gave exactly identical results. A large series of ten experiments

made on May 20 shows the full range of diversity and will serve to illustrate all of the points of interest. As the time element is of prime importance in this study it is necessary to give readers as a norm for purposes of comparison a time schedule of the development of the normally fertilized *Asterina.*

Time Schedule of the Development of Normally Fertilized Eggs of Asterina.

Hours after Shedding.	Condition of Maturated and Fertilized Eggs and Embryos.
2¼	Fertilization membranes formed on majority of matured eggs.
3½	Cleavage beginning.
5	Cleavage taking place in all fertilized eggs, a few 4 and 8 cell stages.
7	Many stages as advanced at 32 + cells.
9	Most of eggs in early blastula stages, but there are a good many eggs without membranes, in early cleavage stages. These are probably parthenogenetic.
25	Two distinct types of larvæ present: the great majority being typical gastrulæ, swimming up near the surface of the dish, and capable of being readily pipetted off; a small minority of living larvæ variously abnormal and swimming at or near the bottom. These latter are probably, though not certainly, parthenogenetic.
49	Normal larvæ, early bipennariæ, forming enterocoel pouches.
73	Bipennariæ with well-defined ciliated bands.
96	Advanced bipennariæ with mouth, œsophagus, stomach, intestine, anterior and posterior enterocœls, waterpore, etc.

Time Schedule of the Development of Parthenogenetic Eggs of Asterina.

No. of Experiment.	Hours after Shedding.	Condition of Maturated Eggs or of Developing Embryos.
I	7	No membranes and no cleavage.
	9	About 1 per cent. of eggs show distinct membranes, no cleavage.
	24	About ½ of 1 per cent. swimming blastulæ, all subnormal in appearance, irregular in shape or solid. About 2 per cent. early cleavage stages (2 and 4 cell stages).
	48	All larvæ and cleavage stages undergoing cytolysis.
II	7	No membranes and no cleavage.
	9	No membranes and no cleavage.
	24	No larvæ nor cleavage.
III	6½	No membranes and no cleavage.
	8¾	No membranes, but about 1½ per cent. of cleavage stages, mostly fairly regular 2 and 4 cell stages.
	25	Nearly 1 per cent. blastulæ, mostly solid and motionless.

IV........ 6..........No membranes and no cleavage.

8½........About 2 per cent. of eggs cleaving without membrane formation, some as advanced as 8 cells.

25..........Nearly 2 per cent. blastulæ, slightly abnormal and motionless.

V........ 6½........About 5 per cent. of eggs with rather narrow but distinct membranes, no cleavage.

7½........About 1½ per cent. of eggs without membranes in early cleavage stages (2, 3, 4 cells), some quite regular, but the majority more or less irregular.

8½........About 5 per cent. of eggs cleaving, ranging from 2 to 16 cells. Evidently a good deal of cleavage has begun since the 7½ hour observation. A few of the eggs now show wide typical membranes and there are transitional stages between these and the more plentiful type with narrow membranes.

26..........About 7 per cent. swimming blastulæ, some nearly normal, but the majority solid, wrinkled or otherwise abnormal. Eggs with membranes, undergoing black cytolysis.

31..........Many gastrulæ, some with two or more archentera some exogastrulæ, etc.

74..........A good collection of twin larvæ, studied in a subsequent connection. No normal larvæ. All larvæ swimming on the bottom of the dish.

VI........ 6¾........About 4 per cent. of eggs with distinct but narrow membranes, no cleavage.

8¼........About 2½ per cent. of eggs with wide, typical membranes, but no egg with membrane shows cleavage; about ½ of 1 per cent. of eggs showing cleavage stages, ranging from 2 to 8 cells, some quite regular.

26½........A very few larvæ, all subnormal, some motionless, others feebly swimming.

VII........ 7..........No membranes, no cleavage.

9..........No membranes, but about 2 per cent. of eggs in cleavage stages ranging from 2 to 32 cells.

26½........About 1 per cent. subnormal blastulæ, a few swimming.

32..........A very few larvæ undergoing gastrulation; several with two or more archentera.

VIII........ 6½........No membranes, no cleavage.

9..........No membranes, but about 3½ per cent. of eggs in cleavage stages, ranging from 2 to 16 cells.

26..........About 2½ per cent. of larvæ, all subnormal.

IX........ 7..........About 8 per cent. of maturated eggs with narrow membranes; about 3 per cent. of eggs without membranes showing first steps in cleavage, a few having completed the first cleavage.

8½........A few eggs in 4- and 8-cell stages.

30½........A fraction of 1 per cent. of larvæ undergoing gastrulation, and swimming about, the best of them being nearly normal in appearance.

78..........All larvæ dead.

X........ 7¼........About 5 per cent. of eggs with fully typical membranes, no cleavage stages.

9½........Over 50 per cent. of maturated eggs in cleavage stages, without membranes, ranging from 2 to 8 cells. No eggs with membranes segmenting.

31..........About 75 per cent. of all maturated eggs have undergone cleavage without membrane formation, and are in various stages ranging from early cleavage to gastrulæ. Numerous dwarf blastulæ, due to blastolomy; many gastrulæ with plural archentera; solid blastulæ and exogastrulæ in considerable numbers. All were swimming about on the bottom of the bowl. This culture was made the basis of a study of the more advanced development of parthenogenetic eggs and will be referred to in more detail in a subsequent discussion.

Significant Points brought out by the Data Shown in the Above Schedule.

1. Membrane formation occurs in exactly half of the experiments here described. It was observed in from 1 to 8 per cent. of the maturated eggs.

2. The degree of completeness of membrane formation varies greatly in different sets of eggs and in different eggs of a given set. In some eggs the membrane is so little lifted from the surface of the egg as to be scarcely noticeable, but in others the membrane is indistinguishable from that seen in fertilized eggs.

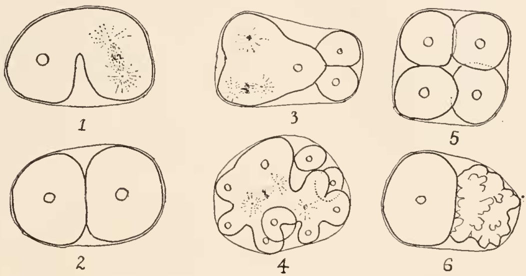
3. Eggs that form membranes, whether narrow or wide, do not further develop, but undergo cytolysis within twenty-four hours.

4. The percentage of maturated eggs that undergo parthenogenetic development varies from none to about seventy-five; in experiment II no cleavage occurred, while in experiment X 75 per cent. of all maturated eggs at least began cleavage. The aver-

age number of parthenogenetic eggs is about two per cent, of maturated eggs.

5. Eggs that undergo parthenogenetic cleavage never form "fertilization" membranes, the closely fitting vitelline membrane being the only envelope that surrounds the blastomeres.

6. Cleavage in parthenogenetic eggs never begins earlier than six and one half hours after the eggs are placed in sea water and the average time for the beginning of cleavage is about seven and a quarter hours. Cleavage begins in fertilized eggs sometimes as early as three and one half hours, and the average time of beginning is about four hours. Subtracting two hours for maturation to complete itself, we have cleavage beginning five hours after maturation in parthenogenetic eggs and two hours after maturation in fertilized eggs. There is, therefore, a retardation in development in the case of parthenogenetic eggs of three hours, and at a very critical period.



FIGS. 1–6. Cleavage stages in spontaneously parthenogenetic eggs of *Asterina*. *1.* An incompletely segmented two-cell stage in which one blastomere is in advance of the other. *2.* A normal two-cell stage. *3* and *4.* Irregular cleavage stages. *5.* A typical normal four-cell stage. *6.* A rather common type of abnormal cleavage in which one blastomere is undergoing cytolysis and the other is remaining normal.

7. Cleavage and subsequent development in parthenogenetic eggs take place much more slowly than in fertilized eggs. Even the most nearly normal parthenogenetic eggs take nearly twice as long to reach a given stage as do fertilized eggs. Development is, therefore, greatly retarded and we would naturally ex-

pect the larvæ to exhibit the various types of developmental defects that are commonly seen in inhibited individuals.

8. In none of the numerous experiments did parthenogenetic eggs give rise to even approximately normal bipennariæ. The most successful larvæ were certain double monsters that will be discussed later.

9. The average viability of parthenogenetic larvæ varies greatly in different sets. As a rule viability was lowest in those sets in which the smallest percentage of larvæ occurred and highest in those in which the largest percentage of larvæ occurred.

10. Individual viability varies greatly within a given set of eggs. Quite frequently eggs die and disintegrate during the first or subsequent cleavages, while it was not uncommon for a few larvæ in each of the best sets to live for from four to seven days.

11. Cleavage in parthenogenetic eggs is sometimes very normal in appearance, but in every set the majority of cleavage stages are irregular (Figs. 1–6). Sometimes blastomeres of the two cell stage separate, and form half-sized blastulæ, seldom going further. In other cases one or more blastomeres cease cleavage while the rest go on and form a covering of small cells about a large central cell. Numerous other cleavage anomalies occur which need not be detailed here.

Discussion.

Loeb's Observations of Spontaneous Parthenogenesis in Asterina.

Doubtless the reader recalls the work of Loeb (1905) on "Artificial membrane formation and chemical fertilization in a starfish (Asterina)." In this paper the author describes various methods employed first, for inducing membrane formation and second, for inducing cleavage and subsequent development in the same species of starfish which forms the material of the present investigation. Loeb recognizes the occurrence of spontaneous parthenogenesis in *Asterina* as is shown by the following quotations: "The eggs of the starfish show a slight tendency to develop spontaneously without any external influence." "If the eggs of *Asterina* are allowed to mature in sea water and are left to themselves, sometimes none, sometimes a fraction of a per cent., some-

times more, will segment and develop into larvæ. But the development of these eggs is much slower than that of fertilized eggs and, as a rule the larvæ are not so perfect and die sooner." "We have, therefore, two types of development in these (*Asterina*) eggs. One type is represented by the fertilized egg, and this type can be produced artificially in a number of eggs, at least, by calling forth the membrane formation by the above-named artificial means. The second type is represented by the spontaneously developing egg in which no membrane has been called forth; these latter eggs begin to segment later, and possibly develop more slowly than the other eggs, and form larvæ which are not as perfect as those belonging to the first type."

It will be seen that Loeb has touched upon some of the essential points that are brought out in my experiments. He notes that spontaneous parthenogenesis occurs in a small per cent. of eggs; that parthenogenetic cleavage takes place without membrane formation; that cleavage begins later; and that development is slower and less normal than is fertilized eggs. Loeb, however, was not primarily interested in the course or results of spontaneous parthenogenesis, but merely dealt with it incidentally as a check upon his work on artificial parthenogenesis or chemical fertilization. He, therefore, merely points out the foregoing particulars without entering into any discussion as to their significance.

Spontaneous Membrane Formation.

In only one important point is there lack of essential agreement between his results and mine: he failed to note any cases of spontaneous membrane formation which was so frequently noted in my experiments. I am at a loss to explain this discrepancy between his results and mine, both performed at Pacific Grove and both unquestionably safeguarded against accidental error. Possibly the material behaves differently at different times of the year and Loeb's work was done at quite a different time from mine, which was confined to the last few days of April and the first three weeks of May. The only difference in treatment between Loeb's cultures and mine had to do with the methods of

sterilizing the sea water in order to avoid normal fertilization. Although he does not mention the fact in these particular experiments, his practice was to use boiled or highly heated sea water; while I used sea water that had been kept in a demijohn for at least four days. It seems barely possible that heating of the water prevents spontaneous membrane formation. I would, of course, have tried this experiment, had I known of Loeb's detailed paper at the time of my experiments, but I had with me only his book on "Artificial Parthenogenesis and Fertilization" and in that book he fails to mention the occurrence of spontaneous parthenogenesis in *Asterina.* Heated sea water would doubtless be relatively poor in oxygen and this might be responsible for his failure to find spontaneous membrane formation. It is possible also that this process (membrane formation) was so belated in its appearance that it occurred after Loeb had ceased to look for it in his cultures. Unless one gets a very early start in experiments with this material the working day is likely to be over before any signs of membrane formation appear, and the next morning these eggs will have undergone cytolysis and their membranes will have disappeared. My plan was to make a before-breakfast expedition to the collecting grounds, get the material ready for work, breakfast, and be ready for experimentation by 8:00 A.M. If one begins to collect after breakfast it is likely to be nearly 11:00 A.M. before experiments with *Asterina* eggs could be commenced. If such were the case it would be 6:00 P.M. before distinct membranes would be visible, later than the time when the investigator habitually "knocks off for the day." It is barely possible then that Loeb may have missed spontaneous membrane formation in some such way as this.

Of the validity of my obervations there seems to be no doubt. In answer to the criticism that failure to heat the sea-water vitiates the observations, it may be said that if these eggs with membranes are fertilized, they should segment; but they never do. We seem to be forced to the conclusion, therefore, that membrane formation in *Asterina,* which Loeb has been at such pains to bring about by chemical means, occurs spontaneously in a considerable percentage of cases. This being true, the various manipulations

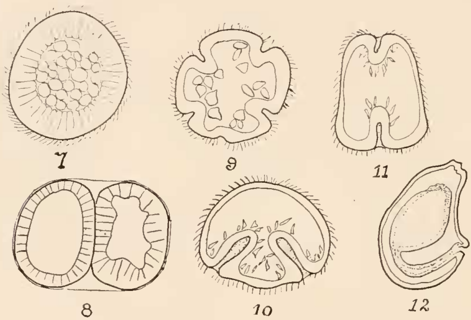
used by Loeb have merely served to hasten a natural process and to cause it to occur in a larger percentage of eggs.

Something in addition to membrane formation occurs in the eggs handled by Loeb, for they go ahead and segment as do normally fertilized eggs, while the eggs that form spontaneous membranes do not segment. From this it would appear that *the so-called "fertilization membrane" is not an essential feature of development, but merely its usual accompaniment.* Thus the voluminous literature dealing with artificial membrane formation, as though it were the most important event in the initiation of development, loses some of its force. Loeb himself recognized that development, at least in *Asterina,* could proceed without membrane formation; witness his statement, corroborated by my own observations, that spontaneous parthenogenesis proceeds without the preliminary of membrane formation. He seems to suggest, however, that this is not to be considered as typical development, since it begins later, goes more slowly and results less normally than in the case of fertilized eggs. Exactly similar results may be obtained, however, in fertilized eggs by the use of agents that retard development, such as cold, hybridization, anæsthetics, etc. So we must admit that *real development may occur without membrane formation, and that membrane formation may occur without initiation of development. The two processes are independent though they usually are associated in normal ontogeny.*

The Development Spontaneously of Parthenogenetic Eggs of Asterina.

According to Loeb, the development of the chemically fertilized eggs differs from that of the spontaneously fertilized eggs in two respects; first, in forming membranes; and second, in beginning earlier and proceeding more rapidly. With this distinction I fully agree. Evidently, in the chemically fertilized eggs, something in addition to membrane formation takes place, a something that results in prompt initiation of the changes expressed by cleavage. In the spontaneously parthenogenetic eggs, however, initiation to development is very slow in beginning, and

is less effective when it does begin. In last analysis the difference is evidently to be expressed in terms of rate of change. *The whole process of ontogeny in these eggs is from the beginning retarded, and the results are exactly similar to those which may be obtained by the use of growth-retarding agents applied to newly fertilized eggs.* The first effect of pronounced retardation of the normal growth process in the egg is the partial or complete obliteration of the characteristic axes of polarity and symmetry in the egg, a breaking down of the axial gradient. This results subsequently in loss of unity of organization, involving physiological isolation of blastomeres or of cell aggregates, in double and triple polarity and consequent double or triple monsters, and in a whole series of products of differential inhibition, such as those described by Child for sea-urchin.



FIGS. 7–12. Later developmental stages in spontaneously parthenogenetic eggs of *Asterina*. *7.* The commonly occurring solid blastula type. *8.* A pair of twin blastulæ enclosed within one vitelline membrane, evidently the result of physiological isolation of two blastomeres in the two-cell stage. *9.* A multipolar embryo gastrulating at several points. *10.* Double monster with two symmetrical archentera. *11.* Another double monster with an additional anterior archenteron. *12.* A microcephalic ciliated larva, with differentiated stomach and intestine, but no anterior parts.

Some of the types of inhibited larvæ found in cultures of spontaneously parthenogenetic *Asterina* eggs are shown and described in figures 7–12. The solid blastula is the commonest type, a type devoid of an axis of polarity (Fig. 7). Forms that are bipolar

and tripolar, etc., usually undergo gastrulation in two or more places (Figs. 9, 10, 11) and produce double and triple monsters, etc. The most nearly normal forms are decidedly abnormal early bipennaria larvæ in which the anterior parts are relatively inhibited. Such a form is shown in Fig. 12, in which the mouth never breaks through, oesophogus is not clearly differentiated, but stomach and intestine are well devevloped. Since I have in preparation a detailed paper on twinning in *Asterina,* in which I intend to discuss the physiology of twinning in general, I shall not enter further into an account of the various types of inhibited larvæ that result from spontaneous parthenogenesis, for the same types result from several other kinds of inhibiting factors. In closing I merely wish to emphasize this one point: that *the results of spontaneous parthenogenesis are those usually found to accompany early growth retardation.* For a summary of this paper the reader is referred to the eleven points referred to on pages 110–112 and to the italicized clauses in the general discussion.

I am greatly indebted to the Hopkins Marine Station of Leland Stanford University, and to its director, Dr. Walter K. Fisher, for the excellent facilities for research that I enjoyed while at Pacific Grove.

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AUTHOR'S ABSTRACT OF THIS PAPER ISSUED
BY THE BIBLIOGRAPHIC SERVICE, FEBRUARY 7

ON THE OCCURRENCE OF PAIRED MADREPORIC PORES AND PORE-CANALS IN THE ADVANCED BIPENNARIA LARVÆ OF ASTERINA (PATIRIA) MINIATA TOGETHER WITH A DISCUSSION OF THE SIGNIFICANCE OF SIMILAR STRUCTURES IN OTHER ECHINODERM LARVÆ.

H. H. NEWMAN.[1]

Introduction.

Ever since the theory became current that the bilaterally symmetrical larvæ of echinoderms afford phylogenetic evidence that this group of radially symmetrical animals was derived from bilaterally symmetrical ancestors, larvæ that showed more than the normal tendencies toward persistent bilaterality have had a special significance.

Normally, the first evidence of the encroachment of the adult radial symmetry upon the the larval bilaterality is seen in the development of a distinct hydrocœl with a madreporic pore and pore-canal on the left side, and none on the right. This failure of the right side to keep pace with the left has been considered as the mechanical cause for the twisting around of the serially repeated primordia of the radial water canals and the assumption of the adult radial symmetry.

The occurrence, therefore, in the larvæ of at least two classes of echinoderms, of paired right and left madreporic pores, pore-canals, and other derivatives of the hydrocœls, looks like the persistence of the ancestral bilateriality and tends to strengthen the current theory as to echinoderm phylogeny.

As early as 1892 Field, in his account of the larval development of *Asterias vulgaris,* described the transitory appearance in all

[1] From the Hopkins Marine Station of Leland Stanford University and the Hull Zoölogical Laboratory of the University of Chicago.

larvæ aged about three and a half days of a strictly bilaterally symmetrical condition of the hydrocœls, madreporic pores, and pore-canals. Soon after this period the right madreporic pore closes and the pore-canal, without an external opening, persists for a short time and then entirely disappears.

Several continental writers had observed the occasional occurrence in asteroid larvæ of paired madreporic pores, but had considered the condition as a pathological one. Field, however, maintains that there normally occurs, in *Asterias vulgaris* at least, a transitory stage in which bilateral madreporic pores exist, and considers this condition as "a true ontogenetic character, and of very considerable phylogentic significance." Gemmill (1912) was able partially to confirm Field's observations of the occurrence of paired water pores in the genus *Asterias,* finding this condition in fifty per cent. of larvæ of *Asterias glacialis* and in about ten per cent. of those *Asterias rubens.* He also states that in all cases the water-pore soon closes.

Paired echinus-organs, madreporic vesicles, and other derivatives of the right hydrocœl, were observed by MacBride (1911) in two very advanced Plutei, one of *Echinus miliaris* and the other of *Echinus esculentis.* A similar condition was observed by Grave (1911) in a single Pluteus of the sand-dollar *Mellita pentapora,* though the figure shows the two madreporic canals making exit through a single median dorsal madreporic pore.

Paired Madreporic Pores in Asterina Larvæ.

During the months of April, May and June, 1920, I had occasion to observe the development of a very large number of cultures of larvæ of *Asterina* (*Patiria*) *miniata* at Pacific Grove, California. In one culture consisting of otherwise normal, healthy larvæ three weeks old, I noticed a larva with two perfect madreporic pores and pore-canals. Further search, involving a complete census of all the larvæ in the culture, revealed twenty-six more larvæ with right madreporic canals in some cases as well developed as the left; but in others with the right pore closed and the pore-canal smaller than the one on the left. More than half of all the advanced larvæ in this culture had the double madre-

poric pores or at least double pore-canals, while the remainder of the larvæ were quite typical. After a prolonged search in other cultures of *Asterina,* I was unable to discover any other larvæ with double madreporic pores.

These double-pored larvæ were carefully watched and attempts were made to rear them by introducing diatoms collected from

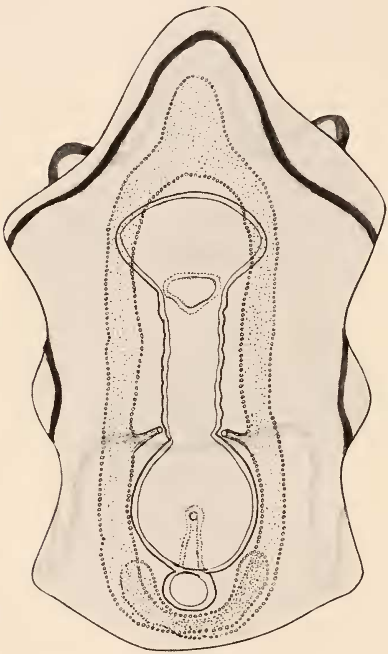
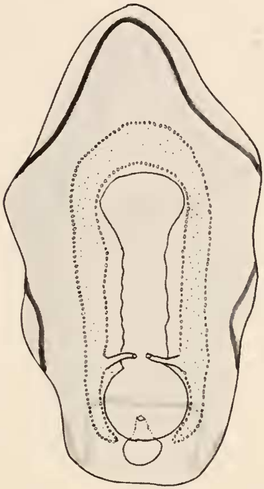


FIG. 1. Dorsal view of largest bipennaria, viewed as a transparent object. The hydroenterocoel cavities are shown stippled. Note both left and right madreporic pores, opening far apart; distinct pore-canals on both sides.

the kelp found in the tide pools inhabited by *Asterina.* They fed to some extent on several species of diatoms, but made no progress beyond the condition in which they were when the double-pored character was first noticed. Instead, they slowly retrogressed in

differentiation, became sluggish, and died without having begun to metamorphose. Throughout their lives they remained bilaterally symmetrical and observations made at the end of the fourth week, shortly before the culture began to die out, showed that the right madreporic pore was still open in several of the largest bipennariæ. While the larvæ were still active and in good health, several specimens were quieted with chloretone and mounted for



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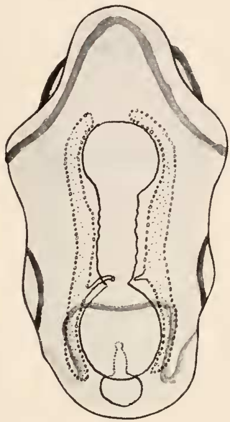

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FIG. 2. Dorsal view of a somewhat less advanced bipennaria with madreporic pores close together but both open.

FIG. 3. Dorsal view of one of the least advanced bipennariæ with right madreporic pore closed, but with distinct pore canal on the right side.

microscopic study. These were drawn with camera lucida and three of these drawings are shown in Figs. 1–3. The largest larva in the culture is shown in Fig. 1. Its length was a little over 7 mm. The anterior hydroenterocœl pouches have become completely fused in front of the oral funnel and have grown forward into a median pre-oral cœlomic cavity. The right and left body cavities are equally well developed throughout and each has grown posteriorly beyond its own side and has curled round

the intestine, so as to have its blind end pointed forward on the opposite side of the body. Thus these two posterior outgrowths cross one another and encroach on each other's territory, a condition that could not well represent any ancestral state, but one to be expected in a double monster such as I believe this to be. Each madreporic canal connects broadly with the dorsal wall of its respective hydrocœl, slopes slightly toward the median line, and opens by a distinct pore to the exterior. The two pores are not very close together in this specimen. The other two larvæ are a little smaller and less advanced than that shown in Fig. 1. That shown in Fig. 2 is about 6 mm. in length, has no pronounced forward growth of the preoral cœlom and does not show an overlapping of the posterior extensions of the right and left cœloms. The pores of the two pore-canals are very close together and might form one double madreporite. The specimen shown in Fig. 3 was of about the same stage of advancement as that in Fig. 2 but differs primarily in the fact that the right madreporic pore is closed and its canal is smaller and shorter than its left partner. Also the anterior cœloms have not fused.

In discussing these anomalous larvæ with Dr. Walter K. Fisher, director of the Hopkins Marine Station, I was interested to learn from him that he had, while collecting, noted adult specimens of *Asterina,* and of other species of asteroids, in which there were two madreporic plates and, correspondingly, two stone canals. This information immediately suggested to me the strong probability that these adults with paired madreporic plates must have arisen from larvæ with double madreporic pores such as I had under observation. The question would then arise as to whether this double condition in the adults would bear the same phylogenetic interpretation as has been offered for the double condition in the larvæ. If not; why not? But this involves us in a discussion of the significance of these anomalous paired structures in echinoderms.

Discussion.

Three distinct interpretations have been offered for the appearance of these anomalous right-hand elements that normally appear only on the left.

1. The first interpretation is well phrased by MacBride[1] as follows:

"*The appearance of a right and left madreporic pore is the first indication of what is really the key to the understanding of Echinoderm development, viz., the fact that the two sides of the larvæ originally gave rise to precisely similar organs, but that some of these organs grew and developed on the left side while they atrophied on the right, and that thus an asymmetry was produced.*"

This seems to be the natural interpretation of the facts, but there are certain other facts that this interpretation does not cover. In a larva of the sand-dollar *Mellita pentapora,* Grave (1919) found that not only were the mesodermal structures, including hydrocoel, pore-canals, etc., bilaterally repeated, but also paired ectodermal pouches occurred which had no reference to the water-vascular system, but were the primordia of the nervous system. Grave is inclined to doubt the validity of interpreting this extra ectodermal pouch as a reversion to an ancestral condition, and I would fully agree with him. The occurence of adult starfishes with paired madreporic plates and stone canals also weakens the phylogentic interpretation of double pores in larvæ; for they are evidently strictly homologous structures. If the double-plate condition in the adult is to be interpreted as a reversion, of what is it an ancestral reminscence? Surely not of an ancestral starfish with radial symmetry of other organs, but bilateral madreporic structures!

2. The second interpretation of paired madreporic structures involves the idea that they are homœotic variations, in Bateson's sense. Such variations may be viewed according to Grave "as cases of perfected symmetry, the result of a long continued strain due to imperfect balance, either morphological or physiological or both, between the organism and its environment." I confess that I am unable to see the force of this interpretation. It is highly mystical in tone and savors of some internal perfecting principle or "entelechy." If such a principle were operative, the real problem would be to account for the failure of echinoderm

[1] "Text-book of Embryology," Vol. I., p. 466.

larvæ in general to continue to maintain their original bilaterality. That is a different problem altogether.

3. A third theory involves the idea that these duplicated structures are the products of twinning and this is what I believe they are. In laboratory cultures of *Asterina* (and in at least two other species of asteroids with which I have worked) there are numerous instances of twins and double monsters. The exact cause of twinning and doubling is not fully known and will not be discussed here, as I have in preparation a more extensive paper on this subject. It may be said, however, that a long series of types has been found in which the original right and left primordia of the future embryo become more or less completely physiologically isolated, and in proportion to the duration or completeness of the isolation, each half develops more or less independently of the other. The result is a series of more or less completely doubled larvæ, as follows: twin blastulæ which soon separate and develop into half-sized larvæ; gastrulæ with paired archentera; gastrulæ with paired blastopores, but with archentera fused anteriorly; early bipennariæ with the anterior end of the archenteron branched and with the beginning of a double set of hydroenterocœl pouches; and finally advanced larvæ with double madreporic pores and water canals. I have placed the type of anomalous larvæ under discussion at the end of what appears to me to be a logical series, representing the results of varying degrees of physiological isolation of bilateral halves of embryos. The type of result attained seems to depend on the time of incidence of the cause or causes of the physiological isolation in question and upon the degree of severity or duration of the causal agent, whatever it may be.

There is therefore no more justification for the use of "double-pored" larvæ of echinoderms as evidence of an ancestral condition than there is for giving a similar significance to instances of dicephaly or spina bifida in vertebrates. For they are all, in my opinion, phases of "twinning" in the broad sense and will doubtless be explicable on some general physiological basis that we shall hope to discuss in the future. Let me close with a word of caution. Beware of giving a phylogenetic interpretation to an

anomaly found in laboratory-bred larvæ of any sort. Such aberrations are more than likely to be the result of subnormal conditions and nothing more.

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Vol. XL. March, 1921. No. 3

BIOLOGICAL BULLETIN

DIFFERENCES IN VIABILITY IN DIFFERENT TYPES OF REGENERATES FROM DISSOCIATED SPONGES, WITH A NOTE ON THE ENTRY OF SOMATIC CELLS BY SPERMATOZOA.

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New College, Oxford.

Having occasion recently to go over the notes on which a previous paper of mine[1] was based, I came across one result which, although not published at the time, now seems worthy of record.

In the paper referred to, it was shown that by filtering chopped Sycons through coarse instead of fine gauze, and pipetting off the first-deposited portion of the cell-sediment, masses of cells could be produced consisting entirely or almost entirely of choanocytes.[2] The unpublished data concern the occurrence, in cultures of such collar-cell masses, of apparently normal regenerating masses similar to those obtained by straining through fine gauze.[3] This would appear to indicate that the chemotactic or other attraction exerted by amœbocytes and dermal cells upon each other is stronger than the similar attraction exerted upon these same cells by choanocytes.[4] This would lead to the observed differential separation of the bulk of the dermal and amœboid cells in preparations where they were present only in very small relative number.

These *normal regenerates*, as they may be called, in opposition to *collar-cell masses*, were found in four of my twelve cultures

[1] Huxley, *Phil. Trans. Roy. Soc.* (*B*), Vol. 202, 1911.

[2] *Ibid.*, p. 177.

[3] *Ibid.*, pp. 167–170.

[4] *Ibid.*, p. 167.

of collar-cell masses. In every case, they lived longer than the collar-cell masses.

The time that elapsed before all the collar-cell masses (whether spheres or blown-out masses) in any one of these four cultures had shown the first sign of impending death by contracting, was from 6 to 12 days; the time before all the masses of a culture were dead, from 8 to 15 days. The time which elapsed before the normal regenerates in the same culture died, however, was from 14 to 33 days; in most cases, all normal regenerates lived longer than any collar-cell mass in the same culture. Whether this greater viability of the masses containing all kinds of cells in approximately normal proportions was due to a protective function exerted by the dermal cells after their migration to the exterior, or to the fact of some dermal or amœboid cells serving as food for the rest, or to other possible causes, remains to be seen. In any case, the facts are interesting.

A further observation may be referred to. It appears that the spermatozoa of calcareous sponges have very rarely been observed. Dr. Gatenby, of University College, London, who has been working on the fertilization of sponges, was discussing the subject with me, when I recalled that bodies resembling spermatozoa had been visible in some of my preparations of normal regenerating masses from dissociations.

Some of my slides I lent to Dr. Gatenby, who re-stained them, and was thus able to discover certain interesting facts. The facts are briefly as follows: Round the margin of all cell-masses from some of my experiments are to be seen minute deeply-staining bodies resembling spermatozoa with an ordinary elongated head; and groups of such bodies are usually to be seen in the interior of the preparations. They are, however, totally absent from other slides representing other experiments. There can be no doubt that these are spermatozoa. The interesting point about them is that they swarm round the masses of cells whether these contain oöcytes or not. *I.e.,* such sponge spermatozoa are attracted by somatic cells as well as by their proper partners.

On re-staining, Dr. Gatenby found in the interior of many of the somatic cells bodies which could be interpreted as heads of spermatozoa which had been half converted into vesicular nuclei.

Here, however, it is impossible to give full proof of this until further material can be examined. But it is at least suggestive that Dr. Gatenby himself[1] has found in the normal fertilization of a closely-related sponge that the spermatozoa do not penetrate the oöcytes directly, but enter collar-cells. Within these they undergo a partial transformation to vesicular nuclei (at this stage closely resembling the bodies found in the cells of the regenerating masses), and are then transferred, by the migration of the collar-cells, to the oöcytes, into whose substance they pass. Within the maturing ova they undergo the remainder of the transformation to male pronuclei, and then effect fertilization in the usual way. If the bodies within the cells of the regenerating masses do prove to be what they appear to be, namely, half-transformed sperm-heads, two interesting points emerge. The first is that somatic cells can exert an attraction on spermatozoa comparable to that exerted by oöcytes. In normal fertilization, only collar-cells within a certain radius are entered by spermatozoa; thus it might be supposed that the attraction was exerted by the oöcyte *through* the collar-cells, and that these had no attraction of their own. That this is not so, would be proved if our interpretation of the bodies in the regenerating masses is correct. But we would have to suppose that this attraction of the collar-cells was much less than that of the oöcytes, whose presence thus would prevent collar-cells beyond a certain distance from oöcytes from being entered.

In the second place, the definite but slight attraction of the collar-cells for spermatozoa would be correlated with the definite but partial transformation of the sperm-head to a nucleus within them. This correlation between degree of attraction and degree of nuclear transformation is what would be expected on such a theory of fertilization as Lillie's.

It is to be hoped that any worker having the opportunity to examine ripe sponge spermatozoa will undertake an investigation of the problem raised in this note.

In conclusion I have to thank Dr. Gatenby for his interest and for permission to publish the results discovered by him.

[1] "The Cytoplasmic Inclusions of the Germ-Cells. Part VIII. Fertilization and Gametogenesis in *Grantia compressa*," *Journ. Linnaean Soc.*, 1920.

May 28, 1920.

A MICRO-ELECTRODE AND UNICELLULAR STIMULATION.

I. H. HYDE.[1]

During the summers of 1918 and 1919, while conducting experiments on unicellular organisms and Echinoderm eggs, it was found necessary to construct a micro-pipette that could be more readily and more accurately controlled than could either Barber's[1] pipette or Chambers's[2] modification of the same.

An apparatus was needed with which it was possible, not only to inject definite amounts of fluid, and extract special parts of cells, but also with which these could be electrically stimulated. Such an apparatus was devised, and although it was only in the process of being perfected, nevertheless with it, fluid could be injected and the membrane and other parts of Echinoderm eggs extracted, and these as well as unicellular organisms electrically stimulated. It was possible, for instance, to stimulate different parts of *Vorticella,* and to determine that the contractile substance of the stalk of this special species differed from the contractile substance of the frog's striated muscle fibers, in that, it did not follow the law of "All or Nothing," which was discovered for these muscle cells by Pratt[4] with his very valuable and interesting micro-electrode. The results pertaining to my experiments with these devises will be published in the near future.

At present only the micro-electrode, imperfect though it be, shall be described, in the hope that it will be improved by some one who has the opportunity and is interested in the experimental fields requiring its use.

The micro-electrodes are Barber pipettes, so modified as to be employed for unipolar stimulation. They are glass tubes about twelve centimeters long, six millimeters in diameter, and drawn

[1] From the Woods Hole Marine Biological Laboratory and the University of Kansas.

[1] Barber, M. A., *The Philippine Jr. Sc.*, Sec. Trop. Med., 9, 307.

[2] Chambers, R., *Am. Jr. Phys.*, 1910, p. 189; Biol. Bull., 1918, 34, p. 121.

[3] Pratt, F., *Am. Jr. Phys.*, 1917–1918–1919, Vol. 43, 44, 49.

out to a bent tip, having a lumen of from three or more microns in the active electrode, and five or more times this in the indifferent one. The other end of the glass pipettes are more or less bent. If they contain mercury, they have a platinum wire soldered into them, and are fitted airtight with rubber tubing and clamps. The rubber tubing and clamp are supported on a pulley that allows this end to be raised or lowered, thus aiding in the adjustment of the mercury in the bent tip.

The pipettes are supported and regulated on the microscopic stage of the Barber pipette holder, and their tips are operated inside of a Barber moist chamber.

The pipettes may contain mercury or some electrolytic solution. Or the indifferent one mercury and the active one a solution, or partly mercury and only the tip a solution. Or both pipettes may contain fine wire. The active one connected with Pratt's glass-coated platinum tip sharpened to a point of 8 microns, and the indifferent one platinum. Or it may be platinum and the active one contain an electrolyte. When non-polarizable, Porter boot electrodes are introduced in the circuit, the pipettes and the dish containing the boots may be filled with sea water. Or by means of pressure applied from the rubber capped end of the pipette, mercury can be forced toward the tip. Then by diminishing the pressure it can be brought back into the capillary a certain distance, drawing after it some of the solution desired from a hanging drop in the moist chamber. An equilibrium is then established that will remain constant as long as the conditions are not changed. Or the active pipette electrode may be filled with mercury either in accordance with Barber's method, or filled under pressure as far as possible and then placed with the indifferent electrode in contact with a mercury hanging drop. If now the circuit from a battery is closed, the current entering the anode traverses the hanging drop and the cathode. At the moment of the establishment of the current, the equilibrium of forces that holds the mercury at a certain point in the capillary is disturbed. The end of the fine thread of mercury moves upward or downward a certain distance owing to a change in sur-

face tension. The direction and distance of the movement depends of course upon the strength and direction of the current introduced into the active pipette. When the mercury is brought to the tip of this, the pressure clamp is closed, the mercury held near the top, which may end in a drop of the same solution that surrounds the organism that is to be stimulated.

If the active electrode be cathodal, a stimulus of a minimal break shock will stimulate, for instance, the stalk or any desired part of a *Vorticella*, that is in the hanging drop, and near contact with the active electrode, and the effect of the stimulation is observed under the microscope.

A diagram of one micro-electrode is shown in Fig. 1. The pipette holder and moist-chamber are omitted in the illustration.

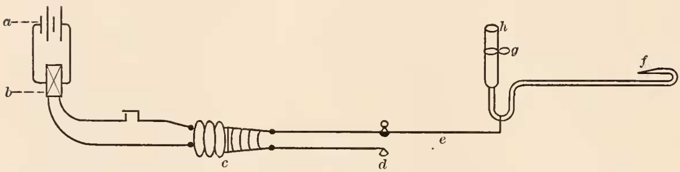

FIG. 1. *a*, battery; *b*, commutator; *c*, induction coil; *d*, clamp; *e*, platinum wire; *f*, tip of pipette; *g*, clamp; *h*, rubber tubing.

The movements of the mercury due to changes in surface tension by a force sufficient to overcome capillary attraction, inaugurated by the passage of an electrical current, made it possible to employ this devise as a capillary electrode.

The fact that the meniscus of mercury moves more or less in either direction in the tip of the micro-pipette by altering the strength and direction of the current, and thus ejecting a solution that lies between the mercury and the tip of the micro-pipette, or drawing in a solution due to suction, led to the idea that it could be adapted for the injection or extraction of minute quantities of substances from unicellular structures. But the mechanism needs further improvements before it can be satisfactorily employed for very accurate work.

I therefore was agreeably surprised while this paper was going

to press, to find that Taylor[1] devised an accurately controllable micro-pipette, that seemed to fulfill the requirements of the apparatus needed for injection and extraction. I believe that by soldering plantinum wire into the capillary containing the mercury and connecting it to batteries, comutator and coil, that it will answer equally well for electrical stimulation.

[1] Taylor, C. V., *Science*, 1920, June 18.

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EFFECT OF VARIATIONS IN OXYGEN TENSION ON THE TOXICITY OF SODIUM CHLORIDE ISOTONIC TO SEA WATER.

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Loeb first observed that he could protect fertilized *Arbacia* eggs for some time from the toxic action of a NaCl solution. isotonic to sea water, by removing the oxygen from the solution or by adding a little KCN.[1] Cyanide also protects unfertilized *Arbacia* eggs against injury by isotonic solutions of various sodium salts, and anæsthetics in appropriate concentrations have a similar effect.[2] Experiments with *Arenicola* larvæ have given similar process being in some manner connected with the destructive results.[3] These results indicate the probability of an oxidative process being in some manner connected with the destructive reaction.

The following experiments were undertaken to determine the effect of varying the oxygen tension on the toxicity of NaCl isotonic to sea water. The form chosen to work with was the larva of the marine worm, *Arenicola cristata,* a free swimming trochophore of three body segments, about ⅓ mm. in length. When normal it is almost constantly in motion, showing ciliary action and bendings to one side, or strong muscular contractions. The larvæ employed were all in the swarming stage and were collected from the lighted side of the culture dish, just below the surface of the water, where they gather in large numbers. As they remain in this stage only from three to four days before developing another segment and sinking to the bottom, a fairly homogeneous culture is obtained. When these animals are placed in pure sodium chloride isotonic to sea water they promptly con-

[1] Loeb, J., *Science*, 1910, XXXII., 411; *Biochemical Zeitschrift,* 1910, XXIX., p. 80.

[2] Lillie R. S., *Amer. Journ. Physiol.,* 1912, XXX., p. 1.

[3] Lillie, R. S., *Amer. Journ. Physiol.*, 1912, XXIX., p. 372, and 1913, XXXI., p. 255.

tract to about ⅔ of their former length and all motion ceases. This contraction is followed by a slow relaxation and eventually they are killed. But if replaced in sea water before death, they slowly recover either completely or to a certain extent. The ratio of those in motion to those still gives an index of the extent of the injury.[4]

Technique.

The larvæ being positively heliotropic, gather in great numbers in the lighted side of the culture dish. A pipette-full (over 500 larvæ) was taken and placed in a watch glass, then, as the larvæ collected again on the light side, the water could be tilted off, the last traces being removed by blotting paper. They were next washed twice with the solution to be experimented with and then transferred by a pipette to 50 c.c. of that solution. Then at hourly intervals about 100 larvæ were removed from the test solution, washed twice with sea water and replaced in sea water. These were inspected one hour and in most cases also twelve hours after their return to sea water. Fifty individuals were examined and the ratio of those in motion to those still was determined.

Experimental.

I. The larvæ were first exposed to 0.52 molecular NaCl in almost complete absence of oxygen. The solution was boiled while a stream of hydrogen was passed through, care being taken

Table I.

Experiment 1.

Over 50 larvæ examined in each case.

Examined 1 Hour after Return to Sea Water.

Solution.	Time of Exposure.	
	3 Hours.	6 Hours.
Isotonic NaCl at atmospheric oxygen tension	No motion	No motion
Isotonic NaCl at reduced oxygen tension	All larvæ show bendings	No motion

[4] For a further description of the behaviour of *Arenicola* larvæ in isotonic NaCl see Lillie, R. S., *Am. Jour. Physiol.*, 1909, XXIV., p. 14, and *Am. Jour. Physiol.*, 1911, XXVIII., p. 210.

to maintain the proper concentration and reduce the temperature to room temperature before introducing the larvæ. This procedure removes the oxygen almost entirely, for if alkaline pyrogallol is added to the solution after this treatment no color appears for an hour or more. The control solution, 0.52 NaCl at atmospheric oxygen tension, was also boiled, air instead of hydrogen being bubbled through.

TABLE II.

EXPERIMENT 1.

Over 50 larvæ examined in each case.

A. Examined 1 Hour after Return to Sea Water.

Solution.	Time of Exposure.		
	1 Hour.	2 Hours.	3 Hours.
Isotonic NaCl at atmospheric oxygen tension	½ show feeble bendings, ½ show no motion	No motion	No motion
Isotonic NaCl at reduced oxygen tension	⅔ show bendings more violent than control	⅓ show bendings	No motion
B. Same Larvæ Examined 24 Hours after Return to Sea Water.			
Isotonic NaCl at atmospheric oxygen tension	1 larva showed bendings. The rest had disintegrated.	All disintegrated	All disintegrated
Isotonic NaCl at reduced oxygen tension	All larvæ actively motile	All disintegrated	All disintegrated

The antitoxic effect of the absence of oxygen is marked. This was to be expected from Loeb's experiments on *Arbacia* eggs.

II. To expose the larvæ to 0.52 mm. NaCl with increased oxygen tension, the simple apparatus pictured was employed. The oxygen tension desired was obtained by running washed oxygen from a tank into the chamber "A," driving the level of water inside the chamber down to a mark previously determined

by calculating the volume required with the necessary corrections. The air already present in the chamber is thus diluted with pure oxygen to the required degree. The water levels in and out of the chamber were kept equal by removing water from the large jar "F." The apparatus is only approximately accurate.

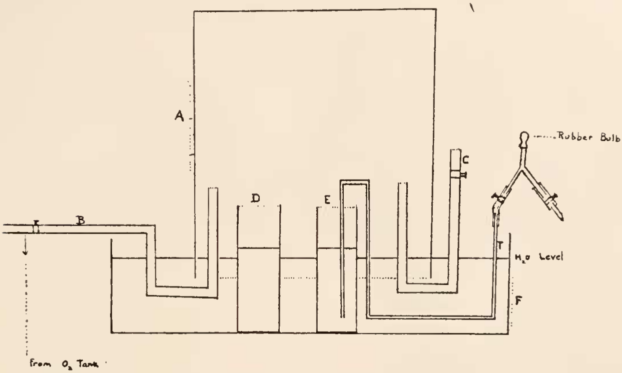

Diagram of apparatus.

The larvæ were introduced and removed by the tube "*T*" of 1 mm. bore, which was connected to a Y tube and the rubber bulb "*H*." By manipulating the rubber bulb and the two pinch cocks on the connections of the Y tube, it was possible to pump the larvæ in or out of the small beaker "*E*." This beaker contained the isotonic NaCl. A similar beaker "*D*" contained a control culture in sea water at the same oxygen tension as "*E*." A third beaker not shown in the diagram contained another control culture in isotonic NaCl at atmospheric oxygen tension.

In the first series of experiments the oxygen tension of the NaCl solution containing the larvæ was raised gradually. The larvæ were placed in the isotonic NaCl solution in the beaker "*E*" before the oxygen tension was raised in the chamber. The tension in the chamber was then raised to 320 mm. and the salt solution and control were slowly stirred for two minutes. The oxygen tension of the solution and control must rise slowly towards 320 mm., but how long it is before it reaches that figure

is difficult to predict. As it was impossible to stir too vigorously for fear of injuring the larvæ and as diffusion is slow across the surface of an unagitated liquid, it seems probable that the oxygen tension of the NaCl solution and the sea water in the control never reached 320 mm. during the experiment.

Table III.

Experiment 2.

Over 50 larvæ examined in each case.

A. Examined 1 Hour after Return to Sea Water.

Solution.	Time of Exposure.		
	1 Hour.	2 Hours.	3 Hours.
Isotonic NaCl at atmospheric oxygen tension	All larvæ show bendings	No motion	No motion
Isotonic NaCl at slightly increased oxygen tension	All larvæ show bendings	1/2 larvæ show bendings	1/4 larvæ show bendings
Sea water at slightly increased oxygen tension	Larvæ normal	Normal	Normal

B. Examined 21 Hours after Return to Sea Water.

Isotonic NaCl at atmospheric oxygen tension	No motion	No motion	No motion
Isotonic NaCl at slightly increased oxygen tension	Larvæ completely recovered	1/10 larvæ show bendings	Occasional larva shows bendings
Sea water at slightly increased oxygen tension	Normal	Normal	Normal

TABLE IV.

EXPERIMENT 2.

Over 50 larvæ examined in each case.

A. Examined 1 Hour after Return to Sea Water.

Solution.	Time of Exposure.		
	1 Hour.	2 Hours.	3 Hours.
Isotonic NaCl at atmospheric oxygen tension.........	1/2 larvæ show bendings	No motion	No motion
Isotonic NaCl at slightly increased oxygen tension...	All larvæ show bendings	1/20 larvæ show bendings	1/10 larvæ show bendings
Sea water at slightly increased oxygen tension.........	Larvæ normal	Normal	Normal

B. Same Larvæ Examined 12 Hours after Return to Sea Water.

Solution.	1 Hour.	2 Hours.	3 Hours.
Isotonic NaCl at atmospheric oxygen tension.........	1/50 larvæ show bendings	No motion	No motion
Isotonic NaCl at slightly increased oxygen tension...	1/4 larvæ show bendings	1/12 larvæ show bendings	1/8 larvæ show bendings
Sea water at slightly increased oxygen tension.........	Normal	Normal	Normal

The toxicity is certainly markedly diminished by the slight increase in oxygen tension. The effect is as marked as when the tension was decreased.

III. In this experiment the oxygen tension of the chamber was raised to 230 mm. four hours before the experiment was started. The isotonic NaCl in the beaker "*E*" was stirred vigorously at intervals of fifteen minutes allowing the solution to come to equilibrium at an oxygen tension of 230 mm. before the larvæ were introduced. The larvæ were washed twice with this NaCl, at 230 mm. oxygen tension, by pumping a few c.c. out through the tube "*T*" and they were then introduced into the beaker "*E*" as

before. The larvæ were therefore suddenly subjected to the change of oxygen tension plus the NaCl.

Table V.

Experiment 3.

Over 50 larvæ examined in each case.

A. Observed 1 Hour after Return to Sea Water.

Solution.	Time of Exposure.			
	1 Hour.	2 Hours.	3 Hours.	4 Hours.
Isotonic NaCl at atmospheric oxygen tension 158 mm.	1/10 larvæ show bendings	1/5 larvæ show bendings	1/5 larvæ show bendings	No motion
Isotonic NaCl at approx. 230 mm. oxygen tension........	All larvæ show bendings	9/10 larvæ show bendings	1/2 larvæ show bendings	1/50 larvæ show bendings
Sea water at approx. 230 mm. oxygen tension.	Larvæ normal	Normal	Normal	Normal

B. Observed 18 Hours after Return to Sea Water.

Solution.	1 Hour.	2 Hours.	3 Hours.	4 Hours.
Isotonic NaCl at 158 mm. oxygen tension....	1/7 larvæ show bendings	1/100 larvæ show bendings	1/50 larvæ show bendings	1/100 larvæ show bendings
Isotonic NaCl at approx. 230 mm. oxygen tension........	9/10 larvæ show bendings	1/2 larvæ show bendings	3/50 larvæ show bendings	2/50 larvæ show bendings
Sea water at approx. 230 mm. oxygen tension.	Normal	Normal	Normal	Normal

This experiment shows the same diminution of toxicity due to slightly increased oxygen tension.

IV. In this experiment the oxygen tension was raised to 275 mm. before the larvæ were introduced, as in the preceding experiment.

Table VI.

Experiment 4.

Over 50 larvæ examined in each case.

A. Observed 1 Hour after Return to Sea Water.

Solution.	Time of Exposure.		
	1 Hour.	2 Hours.	3 Hours.
Isotonic NaCl at atmospheric oxygen tension 160 mm..	1/4 larvæ show bendings	1/50 larvæ show bendings	No motion
Isotonic NaCl at approx. 275 mm. oxygen tension...	1/4 larvæ show bendings	1/100 larvæ show bendings	No motion
Sea water at approx. 275 mm. oxygen tension.........	Normal	Normal	Normal

B. When observed 24 hours after return to sea water there was no motion in any case except the control in sea water at 275 mm. which was normal.

The parallelism with the control is striking. The toxic and antitoxic effects of the increase of oxygen tension must balance at this tension.

V. In the last experiment the larvæ were exposed to an oxygen tension of 756 mm. (saturated). A stream of oxygen was bubbled through a flask containing the isotonic NaCl for fifteen minutes. The larvæ were washed twice with this solution, then were placed in it and oxygen bubbled through for five minutes more and the flask closed. When some of the larvæ were removed at hourly intervals for return to sea water, oxygen was bubbled through the remaining solution for one minute immediately after.

Table VII

Experiment 5.

Over 50 larvæ examined in each case.

A. Observed 1 Hour after Return to Sea Water.

Solution.	Time of Exposure.		
	1 Hour.	2 Hours.	3 Hours.
Isotonic NaCl at atmospheric oxygen tension 160 mm..	3/4 larvæ show bendings	No motion	1/20 larvæ show bendings

Isotonic NaCl at approx. 756 mm. oxygen tension...	No motion	No motion	No motion
Sea water at approx. 756 mm. oxygen tension.........	Normal	Normal	Heliotropism lost, otherwise normal.

B. Observed 18 Hours after Return to Sea Water.

Isotonic NaCl at atmospheric oxygen tension.........	1/2 larvæ show bendings	1/50 larvæ show bendings	2/50 larvæ show bendings
Isotonic NaCl at approx. 756 mm. oxygen tension...	1/50 larvæ show bendings	No motion	No motion
Sea water at approx. 756 mm.........	Normal	Normal	Some heliotropic, some not heliotropic. all otherwise normal.

The increase in toxicity caused by the excessive oxygen tension is very marked.

The writer wishes to express his thanks to Professor R. S. Lillie under whose direction the problem was undertaken.

Conclusions.

1. The removal of most of the oxygen from the solution markedly diminishes the toxicity of sodium chloride isotonic to sea water to *Arenicola* larvæ.

2. Slight increases in oxygen tension also markedly diminish the toxicity of isotonic sodium chloride.

3. Saturation with oxygen markedly increases the toxicity of this solution.

4. At a tension of approximately 275 mm. the toxic and antitoxic effects balance.

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TRANSPLANTATION AND INDIVIDUALITY.[1]

LEO LOEB.[2]

Introduction.

In this paper I shall report very briefly on a series of experiments which have been carried out in our laboratory by ourselves and our associates and in which the method of transplantation was made use of in the analysis of individuality.

Reactions of tissues serve as indicators with which to judge the interactions of individualities when parts of organisms are transferred into a new soil. Conversely, by means of the reactions of tissues, which we observe under those conditions, we attempt to obtain an insight into the forces which are active between tissues and into their finer biochemical correlations. Upon these forces depends the preservation of the structure of the organism and thus its maintenance. Thus we hope to contribute to the building up of a physiology of tissue in contradistinction to the physiology of organs. In addition we have studied the literature of transplantation, in order to obtain data which permit the comparison of the interaction of individualities in lower and higher animals and thus to determine whether there are indications that a gradual change has taken place in the course of evolution. We shall include in our consideration fertilization which can be considered as an intracellular transplantation.

Transplantation is the separation of a piece of tissue from its normal surroundings and its transfer into a new environment, either at a new place in the same host; this we call "autotransplantation"; or into a related individual: "syngenesiotransplantation"; or into a not related individual: "homoiotransplanta-

[1] A lecture delivered at the Marine Biological Laboratory, Woods Hole, on August 3, 1920. A few minor additions to the manuscript have been made subsequently.

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tion." Within the same species differences of groups and strains, varieties may arise.

Transplantation into a different species we call "heterotransplantation." Here, nearly related species, which interbreed, further distantly related species, which do not interbreed, species which belong to different genera, families, classes can be distinguished. To this spectrum of relationships corresponds, with certain limitations, a spectrum of interactions of tissues after transplantation.

We judge individuality in man primarily by characteristic social-psychical reactions. It is, therefore, I presume, originally a term connoting psychical attributes. Each individual is supposed to form an indivisible whole. It is one organism separate and distinct from all others. No two organisms can be identical. This finds the sharpest expression in the conception of a soul which is supposed to be the real bearer of the individuality, which is unlike any other soul and indivisible.

To the psychical individuality corresponds individuality of the body. The body also is supposed to represent one indivisible whole, different from every other body; in short one common factor uniting all the parts, and distinguishing them from all the parts of other individuals.

This conception, however, does not quite harmonize with the mosaic such as has been revealed by Mendelian analysis of inheritable characteristics of organisms. Mendelian analysis has shown an organism to consist of a very great number of unit factors, the various unit factors being approximately the same in many organisms of the same species, and the individuals differing from each other in the mosaic of unit factors of which each one is composed. A common factor or set of factors present in all parts of the organism and truly representing its individuality is not provided for in this scheme.

Individuality Differential.

And yet, such a characteristic present in all parts does exist. It is the same everywhere in the same organism and differs in different organisms. We may call this characteristic individuality differential as far as it distinguishes individuals, and species

differential as far as in addition it differentiates species, the one differential being superimposed upon the other. That such a common factor truly representing the individuality does exist can be shown through a comparative study of transplantation of one part of an organism into the same and other individuals, related and not related, of the same species, and into individuals of various different species. Such transplantations reveal the presence of an individuality differential, either directly through the interaction of tissues and of tissues and body fluids, or in certain cases indirectly through the immunity which follows such transplantation of tissues or parts of organisms.

A systematic use of transplantation for the analysis of individuality is of rather recent origin. Carried on, however, in a more or less haphazard way the study of transplantation dates back a considerable number of years. My own interest in transplantation as a means of analyzing the biochemical difference in the constitution of individuals was first aroused about nineteen years ago, and again a few years later, when I compared the result of transplantation of tumors in the same individual and in another individual of the same species. The transplanted pieces behaved quite differently in both of these cases.

Auto and Homoiotransplantation.

It is, however, only recently that the difference in the result of auto and homoiotransplantation has been more generally recognized. On the other hand, that heterotransplantation does usually not succeed has been established considerably earlier.

The Mechanism which Determines the Interaction of Tissues.

For a number of years my associates and myself have carried out experiments tending to analyze the factors which connect individuality and transplantation, and here I wish to describe very briefly a few of our experiments and to draw some more general conclusions, the latter merely in a tentative manner. Our experimental analysis is not yet concluded and at the present time there are under way certain investigations which we hope will help to clear up some of the doubtful points. For our pur-

pose it might be best to select a few examples of our experiments in order to illustrate some of the more important factors that come into play under various conditions of transplantation. We shall first discuss transplantation of pigmented skin, in the guinea pig, then as examples of glandular organs, the thyroid gland and kidney, and lastly the uterus as an organ containing a variety of tissues, epithelium, myxoid or predeciduomatous connective tissue and unstriated muscle.

Transplantation of Pigmented Skin in the Guinea Pig.

If we transplant black skin of the guinea pig into a defect in white skin of the same individual, the black skin usually heals in and even begins after a few weeks to penetrate into the neighboring white skin. If instead we transplant it into a defect in the white skin of another guinea pig, the skin may temporarily heal in, but sooner or later it is cast off, in the majority of cases at an early date following the grafting. In a few cases, however, the transplant took. I suspect these were cases in which host and donor were related to each other, so that in reality we had to deal with syngensio- rather than homoiotransplantation. Now in these exceptional cases the black skin did not only not penetrate into the neighboring white skin, but on the contrary, it gradually became paler and its pigmentation disappeared in the end entirely. The black skin became transformed into white skin. Microscopically we found in such cases in addition a gradual accumulation of lymphocytes under and in the strange epidermis. The number of these cells need, however, not be very marked. Lymphocytes are small cells with a relatively prominent round nucleus which originate in lymph glands, gain entrance into the circulation and are capable of active movement, which, however, is probably not as active as in the ordinary polynuclear leucocytes. These lymphocytes, as we stated, are attracted by the foreign skin, but not by the skin of the same organism. We notice after this kind of homoiotransplantation, a primary incompatibility between the strange skin and its environment. As a result of this incompatibility between body fluid of the host and the transplant changes take place in the metabolism of the latter which cause the deficient healing in of the transplant and

which later attract the lymphocytes. In addition there occur abnormal reactions in the transplant which render impossible the normal restitution of pigment in the graft. Some of these changes in the transplant may take place so soon after transplantation that the conclusion suggests itself that in this case an incompatibility between the graft and the body fluids of the host interferes directly with those tissue reactions on which depends the healing in of the graft and the reëstablishment of the normal pigmentation. How far these primary changes in metabolism of the graft injure the transplant directly and to what extent they act possibly through induced changes in vascularization of the graft still remains to be determined. It is, however, more probable that the incompatibility between body fluids and graft leads to a direct interference with the transplant.

Transplantation of Thyroid and Kidney

If we transplant thyroid gland into the subcutaneous tissue only the peripheral gland tissue survives, the center of the graft being ill nourished in the first few days following the detachment of the gland from its soil dies. The peripheral gland acini proliferate soon after transplantation. After autotransplantation blood and lymph vessels grow through this ring of well-preserved and temporarily growing thyroid tissue into the necrotic center, and especially in the peripheral part of the latter they form a ring of vessels which is very noticeable; from here vessels penetrate into the center of the necrotic material. They are accompanied by a relatively limited number of fibroblasts which at the inner aspect of the thyroid ring form a loose, almost myxoid connective tissue around the blood and lmyph vessels, a tissue not unlike that found in certain stages of development in the embryo. Further removed from the thyroid ring, in the real center of the necrotic material, there may be produced a nucleus of dense fibrous tissue. But its existence is only temporary after autotransplantation. Sooner or later the blood and lmyph vessels and fibroblasts penetrate into it, absorb it and substitute for it the same kind of loose vascular connective tissue; this again gives way more and more to the peripheral real thyroid tissue. As a result of the absorption of the central connective tissue and of the marked develop-

ment of the peripheral thyroid tissue and of the good vascular supply the normal organ structure is more and more reëstablished. This occurs within three to five weeks after transplantation. Lymphocytes are at no time prominent in the transplant.

After homoiotransplantation of the thyroid gland the first stages are similar to those observed after autotransplantation; but soon two important differences become noticeable. The well developed ring of lymph and blood vessels in the peripheral part of this center is much less prominent after homoiotransplantation; the connective tissue ingrowth, on the other hand, is much more marked. The whole center becomes soon converted into a dense fibrous mass, much larger in volume than in the autotransplant. It may still be well supplied with vessels, but they are less prominent than after autotransplantation. In contradistinction to what happens in the autotransplant this fibrous mass is not absorbed and not substituted first by myxoid and later by thyroid tissue. This transplant never attains similarity in structure with the normal gland. Even around the individual acini the fibroblasts may become active and form fibrillar and fibrous tissue. The activity of the connective tissue may go still further, and occasionally fibroblasts may penetrate into the interior of some thyroid acini and help to destroy them.

The direct destruction of the graft by the host is a very characteristic feature after homoiotransplantation. It is however, not so much the activity of the fibroblasts as of the lmyphocytes which brings about this result. While in the autotransplant the lymphocytes are practically absent, in the homoiotransplant they begin to appear as early as five and seven days after transplantation. However, they become more prominent only about nine or ten days after transplantation, and from then on they rapidly increase in number in many cases. They approach the transplant mainly by way of the lymph vessels, and in consequence in the homoiotransplant the lymph vessels may become as prominent as if they had been injected. This I found especially noticeable in the rat, on account of the peculiar distribution of the lymph vessels at the inner aspect of the thyroid ring. The lymphocytes appear at this place in especially large masses and from here they penetrate in a peripheral direction into the thyroid acini

proper and destroy them. Other lymphocytes approach the thyroid from the periphery and from here push forward in a central direction. All these lymphocytes surround, invade and destroy the thyroid tissue directly after homoiotransplantation and they are, therefore, the chief agent of destruction. They interfere with the nourishment of the acini by surrounding them and cutting them off from the vessels; they exert furthermore a direct pressure upon them and invade and substitute them. In addition the fibrous tissue which is so prominent after homoiotransplantation also tends to shut off the nourishing material from the vessels. Furthermore it compresses the glandular structures and fibroblasts occasionally invade them, as we have stated above. These lymphocytes and fibroblasts form, therefore, an agency of attack which usually succeeds in destroying the homoiotransplant of the thyroid somewhere between the fifteenth and twenty-eighth day following transplantation.

We may again assume that in the strange host the body fluids are not completely adapted to the transplanted gland structures. In consequence their metabolism is to a certain extent altered. This alteration, however, is not of sufficient intensity to cause a direct destruction of the transplanted glandular structures. This alteration in metabolism attracts the lymphocytes, diminishes the vascularization of the transplant and increases the invasion of the graft by fibroblasts, which, however, under these altered conditions do not remain intact, succulent cells, but instead form fibrillar and dense fibrous tissue.

In principle it is similar after transplantation of the kidney; but the greater denseness of this material brings about certain minor modifications in the result. Thus in the kidney after autotransplantation we do not find the inner ring of lymph and blood vessels so noticeable as in thyroid autotransplant. But in all essential respects the conditions are parallel to those after transplantation of thyroid. The formation of fibrous tissue is much more prominent after homoiotransplantation of the kidney than after autotransplantation. The lymphocytes play here a similar part. In the end the homoiotransplant is again destroyed in the same way as in the case of the homoiotransplanted thyroid. But this destruction of the kidney may be completed at a somewhat

later date. After autotransplantation kidney tissue usually remains preserved.

We have begun comparative studies of auto and homoiotransplantation in other species. There are indications that in principle conditions are similar in those species which we have begun to study, but there may exist some quantitative and perhaps also some qualitative differences in different species, just as we found certain differences in the behavior of the thyroid and kidney. Thus to mention only one difference, we found that in the rabbit the polynuclear leucocytes are much more prominent after transplantation than in either rat or guinea pig.

Syngenesiotransplantation.

If instead of transplanting the thyroid gland in the guinea pig into not related individuals of the same species, we transplant it into nearly related individuals of the same family, for instance from brother to brother or sister, or from parents to children, we find an intermediate condition between the results of auto and homoiotransplantation, or rather we find all degrees of an intermediate condition. Thus while after autotransplantation lymphocytes are almost absent and after homoiotransplantation they usually begin to become prominent during the latter part of the second week, they may after syngenesiotransplantation, appear as late as the second half of the fourth week; sometimes they may appear somewhat earlier or at other times later. But they usually end by destroying the transplant even in relatives. We find therefore a noticeable delay in the reaction on the part of the host. Evidently at first the metabolism of the syngenesiotransplant is little altered by the body fluids of the host. Gradually, however, the alteration becomes here also sufficiently strong to attract the lymphocytes. As we have stated above the vascular and connective tissue reaction on the part of the host tissue usually takes place between the seventh and twelfth days after transplantation, at a time, therefore, when the alteration of the metabolism has usually not yet become very marked. The blood and lymph vessel supply may therefore in the case of syngenesiotransplantation be as satisfactory as after autotransplantation, although later the lymphocytes begin their destructive work. There

is a prolonged latent period in the case of syngenesiotransplantation and the vascular and connective tissue reaction falls into this latent period.

As we have stated above we find in syngenesiotransplantation all degrees of intermediate condition in different individuals of the same family, for instance in different brothers. In some individuals host and graft may be almost as unsuitable to each other as after homoiotransplantation. In such a case the metabolic alteration of the transplant may have reached a considerable strength at the period, when the vascular and connective tissue reaction is determined. In this case the dense fibrous tissue of homoiotransplantation may be produced in syngenesiotransplantation.

Most beneficial seems to be the exchange of tissues between brothers and sisters. Very good, but perhaps sligthly less advantageous is the transplantation from parents to children. Peculiarly enough, we found that after transplantation of the thyroid in the guinea pig from child to mother the result is about as unfavorable as after transplantation into a totally strange individual of the same species. This peculiar phenomenon we intend to analyze still further. A similar intermediate condition we found after multiple simultaneous transplantation of organs into relatives in the rat. In this case we have, of course, to take into consideration the fact that different organs and parts of organs differ in their power of resistance in the period following transplantation, and that the relation between the parenchyma and stroma also differ in different organs. Again we found in the latter series all degrees of intermediate condition and different organs behaved in the same host in an analogous way, if they came from the same donor.

Transplantation of Uterus.

In the transplantation of the uterus, we are especially interested in the behavior of the myxoid or predeciduomatous, cellular connective tissue and in the unstriated muscle tissue which are characteristic of the uterine structure. Soon after transplantation the death of a part of the myxoid connective tissue, which is not unlike connective tissue present in the embryo, and of the unstriated

muscle takes place in the auto as well as in homoio graft. This is the result of the injury caused by the process of transplantation and the defective nourishment directly following the grafting. But from about the sixth to the tenth day following transplantation a much better, more complete recovery of the myxoid and unstriated muscle tissue takes place in the auto- than in the homoiotransplant, and while subsequently in the autotransplant these two tissues maintain themselves, in the homoiotransplant a gradual substitution of the injured myxoid and muscle tissue by fibrous tissue takes place sometime between the fourteenth and twenty-fourth day. On this soil of fibrous tissue the epithelium does not thrive so well as on the myxoid connective tissue of the autotransplant; it decreases therefore in size, and becomes lower; later it is again attacked by lymphocytes and thus gradually destroyed. The lymphocytes though appear here somewhat later than in the homoiotransplanted thyroid and kidney; but as in the case of the other organs their attack is mainly directed against the epithelial tissue, although they do not leave entirely free some of the other tissues. Again we find as the result of those abnormal substances which form after homoiotransplantation,—we may call these substances homoiotoxins—an attraction of lymphocytes and an altered behavior of connective tissue cells. But in addition we find in this case a very early injurious influence of the homoiotoxins on two sensitive tissues, namely myxoid or predeciduomatous and unstriated muscle tissue. They show the injurious effect of the conditions prevailing in the strange host at a time, when lymphocytes have not yet had a chance to play any considerable part. How far these two tissues are injured directly by an inadequate constitution of the body fluids of the host (by the "homoiotoxins"), and how far the latter influence primarily the vascular supply, the latter in turn influencing the life of the myxoid and unstriated muscle tissue, is uncertain at present. It is however more probable that their destruction is primarily due to the inadequacy of the body fluids rather than to an insufficient vascular supply, which latter would then be the direct consequence of the lack of adaptation between body fluids and transplanted tissue. There is another point of interest in the transplantation of the uterus. We find that the primary transforma-

tion of myoxid into fibrous connective tissue leads secondarily to changes in the epithelium which rests on the connective tissue. We find thus a chain reaction and in addition to the direct results indirect results of the homoiotoxin action.

Mechanism of the Reactions.

These experiments prove that the introduction of parts of organs or tissues which originated in a strange individual causes disturbances which lead to changes similar to those found as the result of the action of toxic substances. These substances act not unlike those given off by certain microörganisms, as for instance the tubercle bacillus, which cause changes of a not acute character. Lymphocytes are attracted and besides the relations between various tissues are quite markedly altered. We have every reason to assume that these disturbances are due to products of metabolism given off by the introduced tissue, which act as homoiotoxins. We have learned that the action of these substances is graded in accordance with the relationship between donor and host. It is as yet doubtful, how far these disturbing substances are those given off in the normal metabolism of the transplanted cells—substances which are toxic merely because they act on a strange host—and how far they are the product of an abnormal metabolism of the introduced cells, the pathological change being due to the action of the body fluids of the new host upon the strange cells. It is probable that the second alternative holds good at least in many cases. Landsteiner, von Dungern and others have shown that in man certain groups of individuals can be distinguished according to the interaction of blood cells on the one hand, and agglutinins preformed in the blood on the other hand.

While such agglutinins have not been observed in animals, in certain cases it has been found possible through immunization with blood corpuscles belonging to the same species, but to different individuals to produce hemolysins which dissolve corpuscles of the same species and combine especially with the corpuscles of the individual whose blood had been used for injection. These observations, as well as our own experiments to which we referred already, as well as others to be mentioned later render it at least probable that such an interaction takes place between a

constituent of the body fluids of the host and the strange cells and that this interaction leads to the formation of toxic substances. While these toxic substances probably interfere in an injurious manner with certain sensitive tissues and lead to their destruction in an indirect manner, in the case especially of certain epithelial structures they merely alter the metabolism in such a way as would be perfectly compatible with the life of the tissues. But this alteration in metabolism sets into motion secondary processes, in consequence of which lymphocytes, vessels and fibroblasts show an altered relation to the transplanted epithelium and these tissue reactions lead secondarily to the death of the transplant.

Syngenesio and Homoio Toxins as Products of Metabolism.

We have assumed that the substances which are characteristic of the individual and which call forth the reactions which we have described, pass into the surrounding medium as the result of the metabolism of the tissue. In all probability they are not merely decomposition products of proteins. In order to exclude this latter interpretation we studied the behavior of the host tissue towards homoiotransplanted bloodclots in which the blood cells live for a certain period without, however, carrying on an active metabolism. We found that such blood cells do not call forth any of the reactions characteristic of homoiografts; neither lymphocytes nor connective tissue nor vessels behave towards them in any specific way whatever. These homoiodifferentials are as far as we know, common to all the active tissues of an organism. They are the same in the different organs of the same animal. As we stated above, we could show this fact in the rat by multiple simultaneous transplantation of different organs of an individual into the same host. Under this condition all the organs elicit proportionately the same individuality reaction, because the relation between the individuality differentials of host and donor is everywhere the same. Particularly the lymphocytic reaction allows us to estimate this relationship of the individuality differentials in an approximately quantitative way, as our syngenesiotransplantations have shown.

Multiple and Successive Transplantations.

We can demonstrate this fact also by multiple simultaneous transplantations of the same kind of organ, for instance, the thyroid from different individuals into the same host. The lobes of thyroid taken from the same animal behave then in an approximately similar manner, while the lobes taken from different animals may behave very differently, each calling forth a lymphocytic reaction in a quantitative way in accordance with the relationship between host and donor. One piece can call forth a marked reaction, while at the same time and in the same host another piece proves rather indifferent. This indicates that the reaction is of an entirely local character, called forth by the substances diffusing from the transplanted cells into the neighboring tissue. It is not primarily a general reaction of the character of an immune-reaction, which would depend mainly on the presence of substances originating in response to the inoculation of the tissue and carried through the circulation equally to all parts of the body. The local character of the reaction we could prove still in another way, namely by carrying out successive transplantations of the same kind of organ into the same host. Under these conditions the latent period of the lymphocytic and connective tissue reaction was approximately the same after the first and second transplantation. An immune substance which could have accelerated the second reaction had, therefore, not been formed. In certain cases, however, such an immune substance may actually develop and hasten the appearance of a reaction around the transplant. This takes place after inoculation with certain tumors. Thus it comes about that the function of the lymphocytes has been misinterpreted in the case of tumor inoculations. It is believed that they are solely concerned in the production of an immunity against tumor growth. Our observations on the action of lymphocytes in the case of transplantation of ordinary tissues, which date back a considerable number of years, clearly prove that the rôle of lymphocytes is a much more general one, namely, a direct and local, quantitatively graded response to the homoio and syngenesiotoxins. The immunity reaction in certain cases of tumor transplantation is merely a special case in a set of phenomena of much wider biological significance.

Constancy of the Individuality Differential.

This individuality differential is a relatively constant factor that varies not at all or at least to a very limited extent in the same individual after the animal has reached the age, when it is able to obtain its nourishment independently of its mother. Such conditions as pregnancy, temporary undernourishment, certain infections do not suspend the function of the individuality differential. In addition we could show in a separate series of experiments that particularly those changes which call forth compensatory hypertrophy in the thyroid gland do not noticeably interfere with the reaction against the homoiodifferential. The lymphocytes may invade in enormous numbers the gland even after it has become hypertrophic and again in the end they succeed in destroying it.

The Effect of Homoiotoxins on Other Growth Processes.

While thus compensatory hypertrophy does not noticeably modify the action of the homoiotoxins, the homoiotoxins may on the other hand, as our recent experiments have shown, to some extent interfere with the development of compensatory hypertrophy of the thyroid gland, not only by bringing about the destruction of the gland, but apparently also by diminishing the frequency of the hypertrophic changes. In a similar manner our previous experiments had shown that homoiotoxins may to some extent interfere with the production of the maternal placenta and the placentomata such as they can be produced experimentally in the normal as well as in the homoiotransplanted uterus. After homoio-transplantation the experimental formation of placenta is diminished. The homoiotoxins interfere, therefore, to some extent not only with purely regulative processes such as occur after transplantation of organs, but also with those changes which lead to compensatory hypertrophy and to placenta formation. They evidently have an injurious influence on a variety of growth processes, and diminish the intensity of those tissue reactions which initiate placenta formation.

Heterotransplantation.

If we compare with these results of auto, syngenesio and homoiotransplantation, heterotransplantation (transplantation into a strange species) we find some interesting differences. After heterotransplantation tissues generally live only a short time, which varies between a few days or even less and two weeks or slightly more. In a few exceptional cases tissue may even live as long as three or four weeks. For a short period there may be found a slight proliferation of the heterotransplant. But usually the injurious action of the host and particularly of its body-fluids is very marked after heterotransplantation. The quantity of living, well preserved tissue is therefore very much reduced. These differences between the hetero and homoiotransplant are usually quite marked as early as the latter part of the first and the beginning of the second week. There may be added to the direct injurious effect of the host and its body fluids a destructive action of lymphocytes and an invasive action of fibroblasts. But both of these are usually relatively slight; and especially the lymphocytic reaction is markedly less prominent after hetero- than after homoiotransplantation. Fibroblasts and bloodvessels of the host show little activity around the heterotransplanted parenchyma. The vascularization is therefore poor and the number of fibroblasts growing directly around the heterotissue is usually restricted. The connective tissue that does grow has the tendency to form fibrous tissue. At some distance from the transplanted parenchmya the fibroblasts and bloodvessels behave otherwise as if they had to deal with an inert foreign body. While thus the lymphocyte and connective tissue contribute only slightly to the destruction of the heterotransplant—the fibrous tissue exerting an injurious pressure on the heterotransplant—large masses of lymphocytes may collect in the tissue surrounding the heterograft. These lymphocytes, however, are relatively innocuous.

If we ask, how it comes about that after heterotransplantation, notwithstanding the greater strangeness of host and donor, the destructive action of the lymphocytes is not only not more marked than after homoiotransplantation, but on the contrary much more restricted, we may suggest that after heterotransplantation the

grafted tissue is injured to so marked an extent, that a depression in metabolism occurs and the quantity of toxic substances attracting the lymphocytes and produced in the metabolism of the tissue is much diminished. All this applies to the transplantation into not nearly related species. If we transplant into nearly related species, results are much better as has been established by W. Schultz. It seems that in this case the tissues behave almost like homoiotransplants. However it appears probable to me that a comparative study—which so far has not yet been made,—would show that even in this case the results are distinctly less good than after homoiotransplantation. If we disregard the hetero-transplantations into nearly related species, there is after hetero-transplantations not a close connection between the results of transplantations and the relationship of the species used. The heterotransplanted tissues are all so near the minimal threshold which just permits life for a short time, that various secondary factor often become of more importance in determining the duration of life and extent of proliferation of the graft than the character of the species differentials. There exists, however, as we have shown an indication that even here such a relationship enters as one of the determining factors.

Spectrum of Relationships and Interaction of Tissues.

If we now compare the effect of the various kinds of transplantations on the character of the interactions between the tissues of host and graft, we come to the following conclusions:

1. The autotransplants have the greatest degree of efficiency in preserving the integrity of the graft and in maintaining inviolate the boundaries of the organs and in preventing the ingrowth of the connective tissue of the host. The autotransplant behaves in this respect most like the normal organ. From there a decrease in efficiency takes place if we pass to the syngenesio- and to the homoiotransplants. In the heterotransplant this power of preserving the integrity of the graft and of warding off the attack by strange connective tissue has become very slight. There is, however, still noticeable a slight action of the transplanted parenchyma even in this case; but on the whole the tissue behaves not

unlike an inert foreign body. Correspondingly the amount of fibrous tissue formed increases and the extent of vascularization decreases in the direction from autotransplant to heterotransplant. The ability of the transplanted parenchyma to maintain a dominance over the stroma, to keep the fibroblasts intact, and if possible in a myxoid condition and to cause the absorption of the connective tissue stroma decreases in the direction from auto to heterotransplant. Similar is the curve which represents the stimulating power of the parenchyma on the vesselgrowth. It needs further investigation to determine how far the vascular reaction is a primary phenomenon and how far the connective tissue reaction depends upon it. The lymphocytic reaction on the other hand reaches a maximum in the homoiotransplant and decreases again in the heterotransplant.

Theoretical Considerations. Contact Substances, Autosubstances and Toxins.

Our results are best understandable, if we assume that the cells in their metabolism give off a series of substances which regulate the relation with certain other kinds of cells, and in particular the relation of parenchyma (epithelial, glandular, special kinds of connective tissue cells and unstriated muscle) to the surrounding connective tissue, blood and lymph vessels and lymphocytes.

We must conclude that the cells living under what we might call "autocondition," give off another substance or another set of substances from those living under "syngenesio," "homoio," "hetero" conditions. We have found a graded injuriousness of the latter kind of substances for the cells of the surrounding tissues. They stimulate the surrounding tissues to reactions which are pathological and which in the end lead to the destruction of the cells which produce these substances and call forth these reactions. We have therefore called these substances "syngenesio," "homoio," "hetero" toxins. Conversely we may designate those substances which are given off by the normally functioning cells under "auto" surroundings as "auto" substances. They regulate the action of connective tissue cells, vessels and

lymphocytes in the most adequate way, and in a manner peculiar to each organ, in a way which keeps away lymphocytes, which limits the activity of fibroblasts which under those conditions cannot invade the tissue of another kind and merely enter in such relations with these tissues as are demanded by the normal structure and function of the organ. The auto substances thus bring about the restitution of the normal structure in an at first disorganized organ, just as these substances maintain the normal structure and function in the normal organ. The syngenesio, homoio and hetero conditions on the other hand lead to those pathological conditions which we have described. There are other facts which equally point to the conclusion that substances which in general we may designate as "contact substances" are given off and regulate the relations of various kinds of tissue to each other.

We may mention a few examples of conditions under which contact substances come into play.

(*a*) In our study of the cyclic changes in the mammary gland we found a relation between the state of the glandular parenchyma and the surrounding connective tissue stroma. We found that an active gland has an active stroma, while a resting, retrogressing gland has a resting, more or less fibrous stroma.

(*b*) Similarly we find around the various gland ducts which are metabolically inactive usually a resting fibrous stroma, in contradistinction to the cellular stroma around the active gland tissue.

(*c*) Wherever cells of the parenchyma are multiplying we generally find the stroma cells and the vessels to become likewise active and conversely where connective tissue cells and the vessels are active the cells of the parenchyma, for instance, epithelium, receive a stimulus to grow.

(*d*) There are indications that contact substances play also a certain rôle during embryonic life and that here they help to determine the formation of some organs. Thus a lens producing contact substance is given off by the optic disc. It stimulates growth and a special differentiation in the overlying ectoderm.

Thus we know at the present time a large class of contact substances and it is probable that future studies will still add to the number of these substances.[1]

[1] Several years ago Dr. Walsh and the writer found that a substance given off by the ovum determines the development of the follicle in the ovary.

Contact Substances and Hormones; Their Relation to the Individuality Differential.

These contact substances are contrasted with the hormones, for instance those given off by the corpus luteum which regulates the activity of the mucosa of the uterus and probably of certain other organs. They are given off by certain organs, and carried through the circulation to distant organs. They have a specific distant action. In contradistinction to some of the contact substances these distance substances do not possess an individuality or even a species differential.

Contact Substances and Changes in Old Age.

I cannot conclude a consideration of this aspect of the subject without pointing out the similarity of conditions which we find under "homoio conditions" with those found in old age. In both cases we observe a tendency to the formation of a fibrous stroma and to a decrease in good vascularization. This condition also corresponds to that found in states of metabolic inactivity. May we not therefore refer old age changes not only to the lack of certain hormones given off by glands (as for instance thyroid and corpus luteum) with internal secretions, but also to a quantitative or sometimes perhaps even to a qualitative change in the character of contact substances, to a diminution in the production of what we have called the "auto substances"? Such a diminution in auto substances would be the necessary result of a diminished activity of the parenchyma. Thus a vicious circle would be established. The diminished activity of the parenchyma causes changes in stroma and vascularization and the latter further depresses the activity of the parenchyma.

Inheritance of the Individuality Differential.

We have seen that there is in each individual and in each cell, at least in the large majority of all cells of an indivdual, an individuality differential which is present in addition to the ordinary Mendelian unit factors. The inheritance of the latter has given rise to numerous investigations which on the whole have tended to show the general applicability of Mendelian rules in the in-

terpretation of phenomena of inheritance. The concept of the individuality differential is too new to have received much attention from students of heredity. But von Dungern studied the group agglutinins to which we have referred above and mentions that they are inherited according to the rules of the inheritance of simple Mendelian monohybrid factors. G. Schoene likewise inquired how the characteristic of tolerance for skin grafts was transmitted from parents to offspring and this author also came to the conclusion that it was inherited according to the rules of simple Mendelian heredity. Neither of these investigators, however, gives convincing data in this respect nor does Schoene refer to the finer tissue reactions in his interpretations. Our own experiments lead us to a different conclusion. In the union of a female and male germcell of two individuals belonging to the same species, two different individuality differentials are hybridized. How are the individuality differentials of the children? Do they follow that of the father or that of the mother or do the differentials of some children follow that of the father, while those of others follow that of the mother? We find that the individuality differentials of the offspring are not identical—at least in the large majority of cases—with the individuality differential of either of the parents, but that they have an intermediate character; there exists, however, as we have shown, all transitions between one and the other of the two individuality differentials, if we compare the behavior of different children. We have therefore a mode of intermediate or blending inheritance. If we interpret this fact in Mendelian conceptions we must conclude that the individuality differential is represented by multiple factors.

Individuality and Species Differential of Substances. Specifically Adapted Substances.

The cells and tissues of an organism contain, as we have seen, the individuality and species differential; they characterize each individual and distinguish it from all other individuals. They make a real indvidual out of a conglomeration of cells and tissues. In order that these cells and tissues develop into an individual

organism and subsequently function as such, substances are given off by the tissues and organs which act upon adjoining or distant parts of the body and thus bring about a correlation of functions which makes possible the orderly development and maintenance of the organism. Some of these substances still preserve the individuality and species differential. We may, therefore, conclude that not only cells, but also substances given off by cells may still have the individuality or species differential. This applies to some of the contact substances which we have postulated. They may have the individuality differential. In most substances, however, the presence of an individuality differential cannot be demonstrated, but merely the existence of a species differential. Of especial interest among these are certain substances which make use of this species differential in their function and which are therefore most effective if they interact with other substances having the same species differential or rather a supplementary species differential adapted to the first differential. Such substances I have called "specifically adapted substances." To this group of specifically adapted substances belong, as I found in my earlier work, substances which are present in the tissues and erythrocytes and which interact with a constituent of the circulating bodyfluid in order to accelerate the clotting of the blood and lymph. These substances I have called tissue coagulins. They seem to be identical with the thrombokinases. The species differential fulfills in this case a definite and important function. Subsequently Hedin discovered in the gastric juice a substance inhibiting the milk coagulating enzyme. Both these substances interact with the species differential. More recently Dr. Frank Lillie found a specificially adapted relation between a constituent of the egg, an agglutinin, and the spermatozoa of the same species. In this case, however, the common species differential functions in substances belonging to two different individuals, while in the former cases the interaction occurs in substances of the same individual. On the other hand a large number of important substances which have the function to correlate and unify the action of various organs, particularly certain growth substances and the common hormones are not only not

individual specific but not even species specific. This applies for instance to the growth substances emanating from the corpus luteum in which I found the absence of the individuality differential. The lack of the species differential applies to growth substances extracted from the placenta, to the growth substances which determine the formation of the lens, to substances inducing metamorphosis in amphibia and compensatory hypertrophy in mammals; it applies to the common hormones of the adrenal gland and thyroid.

In this case we have to deal with relatively simple substances, some of which are apparently of a lipoid nature, while others are of a still simpler composition. On the other hand we have every reason to assume that the individuality and species differentials are proteid substances or at least that they occur only in combination with proteid substances.

In the case of the tissue coagulins we have found that the species differential of the tissues interacts with an adapted substance in the body fluids. In a similar way we find in general an adaptation between body fluids and cells which is based on the presence of the individuality and species differentials. It can be demonstrated directly in the case of the natural hemolysins, but probably applies as we have pointed out above, to the relations of all tissues to body fluids. It exists, as we found recently, even in the case of invertebrates where we established the interaction of species differentials in the case of the experimental amœbocytic tissue and the blood serum. We shall refer to it again later. It also applies to the relation between the antigens and immune substances.

Supplementary Differentials in Body Fluids.

In all those cases the individuality or species differential of the tissues interacts with an analogous substance in the body fluids. It is, however, not certain that the substance or group in the body fluids is identical with that in the tissues, although they fit into each other specifically. A difference between the two could be demonstrated in the case of tumor immunity. While species immunity can be produced with body fluids as well as with tissues of a certain species, individuality immunity can only be produced

with the tissue differential, but not with the corresponding differential in the body fluids. We may designate the substance present in the body fluid and interacting with the individuality and species differential of the tissues as the supplementary individuality and species differential.

Phylogenetic and Ontogenetic Evolution of the Individuality and Species Differential.

The analysis of the individuality differential which we have given so far applies altogether to the higher animals, mammals and birds. Almost all the experiments to which we referred in this paper were made in these two classes. Does this fine differentiation of individuality, the delicate discernment of individual relationship such as we have found in the cells and tissues of higher animals, also exist in the lower animals? Is it a characteristic of all animal cells or has it gradually evolved together with other differentiations in the course of evolution? Have the differentials, or rather the reactions they call forth, had an evolution like structure and certain functions? So far as I am acquainted with the literature this question has never been put and no planful investigations have been carried out tending to answer it. In a general way, however, it is known that in lower forms and in earlier embryonic stages transplantability is greater, in correspondence with the greater regenerative power of these organisms, or in dependence on the greater power of isolated parts of these beings to sustain themselves separated from the remnant of the animal, as particularly W. Schultz has pointed out. But the evolution of species and individuality differentials has not been considered in a conscious way as far as I am aware.

I have recently studied the literature of transplantation with the view of determining whether the experiments which were carried out by various investigators for the solution of problems of a different character might throw some light on this question. While in some respects I found the evidence here and there somewhat contradictory, still I believe that certain conclusions may be arrived at on this basis with a fair degree of certainty.

To begin with invertebrate embryos, the experiments of Driesch, Morgan, Goldfarb and others show that parts of em-

bryos of the same species can be readily grafted upon each other. The homoiodifferential does apparently not play any rôle. These embryos on the other hand react against union with the part of an embryo belonging to a different species, against an heterodifferential; the reaction, however, seems to be less marked than in mammals. Similar are the results obtained with transplantation of gonads in larvæ of lepidopteræ (Meisenheimer, Kopec). In nearly related species the results of grafting are better than in distant species.

It is similar in adult invertebrates. Here also no difference seems to exist between auto- and homoiotransplantation and while a reaction takes place against heterotransplants, it is again considerably less pronounced than in the higher animals. Of value are here especialy the experiments of Joest, Harms, Leypoldt and Rabes in Lumbricidæ and of Wetzel in *Hydra*. While heterodifferentials and also reactions against heterodifferentials exist here, the reactions are much less intense, if we make allowance for the lower temperature at which these reactions take place and which would naturally retard the reaction considerably. In Lumbricidæ the heterotransplant may remain alive in toto, while even in amphibia part of the heterotransplant becomes necrotic. In adult invertebrates also heterotransplantation succeeds only in certain, not too distant, species. We studied recently the species differential in such simple forms as the blood cells of arthropods. We found that the blood cells of limulus are specifically adapted to their own blood serum. Their activities are optimal in *Limulus* serum. *Limulus* serum surpasses any other serum so far tested.

In order to determine this relationship we made use of experimental amœbocyte tissue and we compared the rapidity and quantity of outgrowth from this tissue in different sera. The pictures we obtain under those conditions are very similar to those presented by outgrowing embryonic connective tissue. We find then even in the blood cells of invertebrates a specific adaptation between the species differential of the blood cells and the supplementary species differential of the body fluids.

If we pass to the lower vertebrates, we note in amphibian larvæ a condition somewhat analogous to that in invertebrates. Espe-

cially the grafting of amphibian embryos, a method originated by Born and subsequently used by Braus, Harrison and others, is interesting in this connection; furthermore the transplantation of skin (Lewis, Weigl), of the eye (Uhlenbath) and the experiments of Spemann add valuable data. There is apparently no difference between the results of auto- and homoiotransplantations. On the other hand a heteroreaction again exists, but is less marked than in the case of higher veretebrates. Transplantation into nearer related species succeeds better than into further distant species.

In adult amphibia we find the first indication of the existence of a homoiodifferential. However, in adult fishes and amphibia the reaction against homoiotransplantation seems to be less marked than in adult mammals and birds, although we must confess the evidence concerning the fate of homoiotransplanted tissues in these classes appears somewhat contradictory. We have furthermore to consider the difference in temperature at which reactions occur in lower and higher vertebrates. This factor might render the homoio reaction much slower in amphibia. Despite these difficulties we may provisionally conclude that the reaction against homoio and heterodifferentials is somewhat less pronounced in lower than in higher vertebrates.

The interesting observations of Murphy and Rous who made successful heterografts of tumors in the allantois of chick embryos suggest that the individuality reaction is absent even in the embryo of higher vertebrates which serve as hosts. This agrees with an observation of Braus which seems to indicate that in amphibian larvæ used as hosts the heteroreaction appears at a certain stage, namely, when the circulation has been established in the transplant. Likewise if we transplant embryonic mammalian tisue into adult hosts the homoioreaction seems to be somewhat elss pronounced in the case of certain tissues; the reaction, however, does exist. Embryonic mammalian tissues call forth a very rapid heteroreaction in adult hosts (Saltykow).

To summarize, we find absence of homoioreaction in invertebrates and larvæ of lower vertebrates. The heteroreaction exists here, but is less pronounced. In lower adult vertebrates the

homoioreaction as well as the heteroreaction exists, but again both are probably less pronounced than in mammals.

There has, therefore, as far as we can judge from the experiments so far recorded, taken place an ontogenetic as well as a phylogenetic evolution in the formation of the individuality differential. Ontogenetic and phylogenetic evolution has apparently led to an individualization of the organisms. There is added gradually to the reaction against the species differential the reaction against the individuality differential.

If we inquire into the mechanism on which this gradual refinement depends, several factors would have to be considered:

1. The individuality differential might as yet be absent in the lower forms, especially in invertebrates, and appear only in vertebrates.

2. The individuality differential might be present in all animal organisms, but the supplementary substance in the body fluids might develop only in certain ontogenetic and phylogenetic stages and consequently the reaction on the part of the host might be very weak.

3. The individuality differential might be present and the host might react towards it, but somehow the tissues of lower organisms show a very low degree of sensitiveness to these reactions. In other words, is this individualization due to the gradual acquisition of the individuality differential or to the development of a reaction on the part of the host against the individuality differential or to a greater sensitiveness on the part of the higher tissues to this reaction? Authors have so far not differentiated between these three possibilities, and the data on hand do therefore not permit us to decide the question definitely.

There exist, however, some facts which have a bearing on this question. In the first place we find a very interesting exception to the statement that individuality reactions are absent in lower forms: in a certain rhizpod. *Orbitolithes,* Max Schultze observed as early as 1863 a peculiar reaction which was further analyzed by P. Jensen. Two pseudopodia belonging to the same individual unite, the protoplasms flowing together at the point of contact; but if two pseudopodia belonging to two different individuals touch each other a contraction occurs and a reunion fails to take

place. An individuality differential seems to be present in these protozoa and to lead to a marked direct interaction of protoplasms. There exist, perhaps, a few similar reactions in certain other unicellular organisms. In other similar protozoa, however, the reaction has not been observed by more recent investigators.

If it were possible to generalize from this observation, we should conclude that the individuality differential is present in all animal tissues; and that the lack of reaction must be due to one of the two other causes. On the other hand there are some indications that the chemical constitutions of the cell proteins of the embryo differ from those of the fully developed forms. To mention only one fact of this kind: while according to Roessle embryonic tissue contains the same antigen for hemolysins as the adult tissue, it lacks according to Braus the substance which after injection into animals of a different species or class calls forth the production of precipitins; neither does embryonic tissue bind precipitin which had previously been produced through hetero injection of adult material. Probably the precipitin reaction is in this case a less reliable indicator of the presence of the differential than the hemolysin reaction, which seems to be positive. It is possible that the absence or presence of the supplementary substance in the body fluids may at least in part, be responsible for the hetero reaction; this is suggested by the observation of Braus according to whom extremities of amphibian embryos grafted on hosts which are as yet in an embryonic stage show the effect of the unsuitable soil, as soon as the body fluids of the host begin to circulate in the grafts.

If we take all these facts into consideration, it seems on the whole more probable that the individuality differentials exist in all animal organisms, but that the individuality reactions are lacking in lower forms.

Individuality and Species Differential and Fertilization.

There exists an interesting correlation between the lack of the individuality differential in lower ontogenetic and phylogenetic forms on the one hand and between the lack of an individuality reaction on the part of egg and sperm chromosomes throughout all classes on animals. Homoiofertilization is the normal occur-

rence and it can be considered as an intracellular transplantation. Homoiotransplantation succeeds in this case as well as autoreaction. Indeed it is a transplantation which usually occurs under homoio conditions. Furthermore, just as in the case of the more primitive tissues a reaction of incompatibility occurs after heterotransplantation, in a corresponding way a reaction of incompatibility occurs, when chromosomes of germ cells with different species differentials meet, as the experiments of Baltzer, Tennent, Moenkhaus, Guyer, Newman and others have shown. With this conclusion is even in agreement the cross-fertilization between widely different classes which have been made possible through the discovery by Jacques Loeb of a method permitting such hybridizations. In this case the incompatible chromosomes are eliminated.

There is an additional similarity between heterotransplantation and heterofertilization. While in both cases the incompatibility increases on the whole in correspondence with the distance of the species, there is no absolute agreement between the effect of heterofertilization and heterotransplantation on the one hand and the relationship of the species on the other hand and in both cases reciprocal relations may lead to very divergent results.

There exists on the other hand, as far as we can judge from a study of recorded hybridizations, one noticeable difference between the reactions of chromosomes and tissues. In the latter there is an indication that the incompatibility between species differentials increases in the course of ontogenetic and phylogenetic development. A study of hybridization on the other hand does not suggest a greater mutual tolerance of chromosomes of different species in invertebrates as compared to those of vertebrates.

Lack of the Homoio Sensitiveness in Certain Mammalian Tumors.

We have so far assumed that all mammalian tissues possess the individuality differential and call forth reactions against the homoiodifferentials. In general this statement is correct. There exists, however, at least one notable exception to this rule. There are certain tumors which are able to grow with the same

readiness in another individual of the same species as in the organism in which they originated. This applies by no means to all tumors, on the contrary the large majority of tumors behave in this respect about like ordinary normal tissues which succumb to the homoioreaction, and are only able to grow in the individual a part of which they formed originally. Why there is a relatively limited number of tumors which behave differently, why they are able to withstand the injurious influences of homoiotoxins which destroy the large majority of tumors, we do not know definitely. It is, however, very probable that these tumors also possess the individuality differential, and that their ability to withstand the homoiotoxins is due to their diminished sensitiveness combined with an increased growth energy. Very often the "homoioreaction" which is ordinarily lacking in these tumors can be called forth through previous immunization of the host.

It is exactly such tumors which lack the individuality reaction which have been used by Tyzzer, myself and Fleisher and again by Tyzzer and Little in order to study the inheritance of tolerance for grafted tumors. Tyzzer crossed for this purpose white and Japanese waltzing mice; Fleisher and myself used diverse strains of white mice. The strain differential is further distant in the spectrum of relationships than individuality differential; and Tyzzer and Tyzzer and Little dealt even with something akin to species differentials. Tyzzer believed that such investigations may throw light on the character and origin of tumors. While this view is probably not tenable, these investigations throw some light on the hereditary transmission of strain and species differentials. I found here again an intermediate mode of inheritance, while Tyzzer and Tyzzer and Little found a more complex mode of inheritance. Again I suggested in interpreting my own as well as Tyzzer's results the presence of multiple factors, an interpretation which was subsequently adopted by Tyzzer and Little.[1]

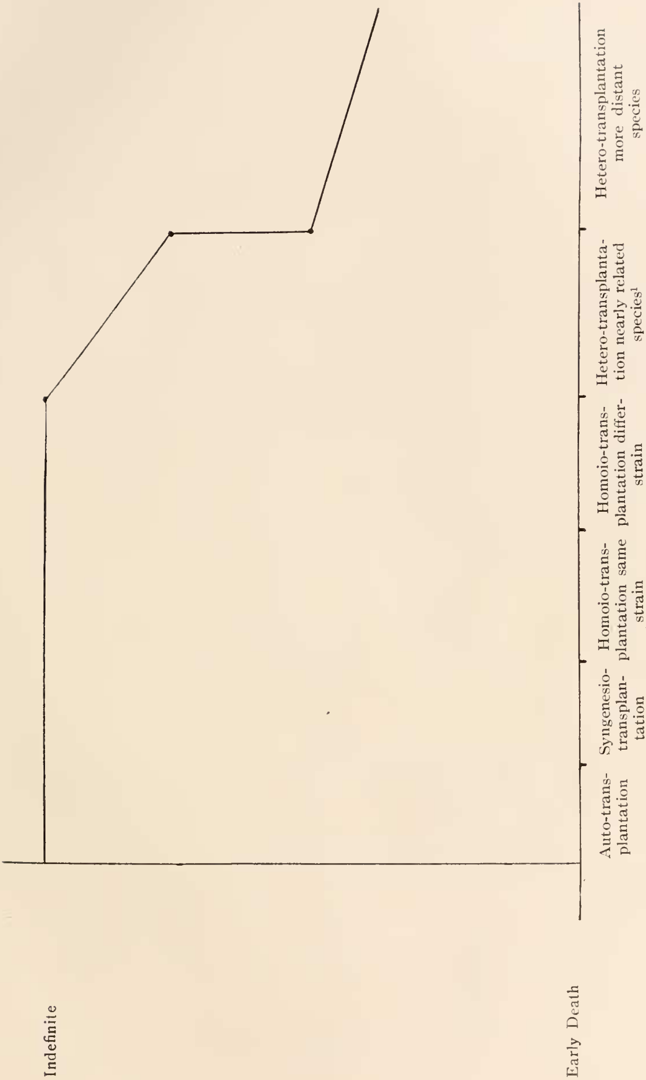
[1] In a paper which just appeared—after completion of this manuscript (C. C. Little, *Journal of Experimental Zoölogy*, 1920, XXXI., 307)—Dr. Little expresses the opinion that the intermediate type of heredity is not the typical mode of inheritance of the individuality differential. I believe that the views which Dr. Little expresses are based on a lack of distinction between species or strain differential on the one hand and individuality differential on the

Individuality Differential and Potential Immortality of Somatic Cells.

The fact that certain tumors can withstand the action of the homoiotoxins has a still wider bearing. We must remember that common transplantable tumors are the direct descendants of ordinary tissue cells, such as we normally find in the individuals of the particular species which we use. The tumors may be derived from a variety of normal tissues and in general the transformation from normal cells into tumor cells takes place under the influence of a long continued action of various factors enhancing growth. Tumor cells are therefore merely somatic cells which have gained an increased growth energy and at the same time somehow gained, in some cases, the power to escape the destructive consequences of homoiotoxins. This ability of certain tumors to grow in other individuals of the same species has enabled us to prove through apparently endless propagation of these tumor cells in other individuals that ordinary somatic cells possess the potential immortality in the same sense in which protozoa and germ cells possess immortality. Thus tumor transplantation made possible the establishment of a fact of great biological interest which because of the homoiosensitiveness of normal tissues, could not be shown in the latter.

We wish, however, especially to emphasize the fact that our experiments did not merely prove the immortality of tumor cells, but of the ordinary tissue cells as well, the large majority or all of which can be transformed into tumor cells. At an early stage of our investigations we drew, therefore, on the basis of these experiments, the conclusion that ordinary tissue cells are potentially immortal; notwithstanding the fact that especially under Weismann's influence the opposite view had been generally ac-

other. I have referred to this distinction in this paper; I have also emphasized how unsuitable transplantable tumors are for the analysis of individuality differentials. In my previous papers I have discussed the influence of such adventitious factors as sex, age and pregnancy on the individuality differentials, and showed that within the limits of our experiments such factors do not noticeably influence the individuality reaction; this applies to guinea pigs above the age of four weeks, as far as the age factor is concerned. We also referred to the apparently abnormal behavior of tissues of the child transplanted to the mother. The factors that are responsible for this peculiarity need, as I stated above, further investigation.



Curve I. Transplantation in invertebrates and embryos.

[1] This part of the curve is only approximate.

cepted, and as it seems to us, with full justification, inasmuch as no facts were known at that time which suggested the immortality of somatic cells. It was the apparently endless transplantation of tumor cells which proved the contrary view.

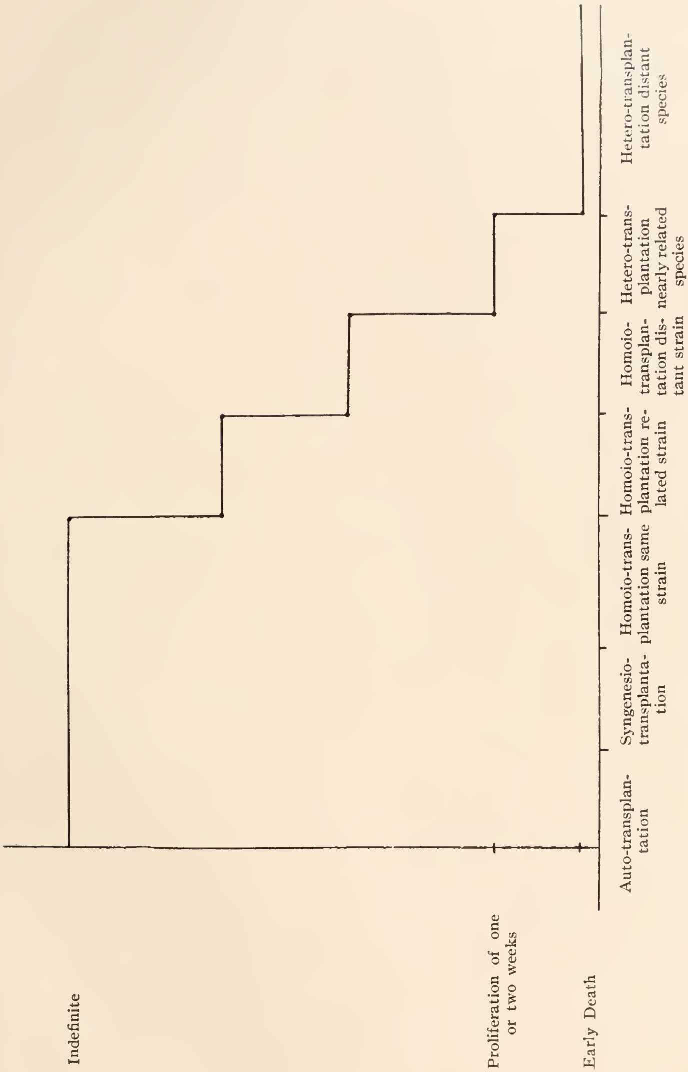
To recapitulate what we stated above: tumors are merely transformed tissue cells. All or the large majority of adult tissues are potential tumor cells. Tumor cells have been shown experimentally to be potentially immortal, therefore tissue cells are potentially immortal.

This wider conclusion I expressed nineteen years ago. Quite recently the immortality of certain connective tissue cells has been demonstrated by Carrel through in vitro culture of these cells. Under those conditions the tissue cells escape the mechanisms of attack to which the homoiotoxins expose the ordinary tissue cells in other individuals of the same species. Under these conditions the reactions of the host tissue against homoiotoxins which would have taken place in vivo, are eliminated. We must, however, keep in mind that this method of proving the immortality of somatic cells applies only to one particular, very favorable kind of cells and it is very doubtful, if by cultivation in vitro the same proof could be equally well supplied in the case of other tissues. On the basis of tumor transplantations on the contrary we were able to show that a considerable variety, perhaps the large majority of all tissue cells possess potential immortality.

Growth Curves and Spectrum of Relationships.

We may approximately represent the effect of syngenesio, homoio, and heterotoxins on various kinds of tissues in the form of curves, where the base lines indicate the spectrum of relationships and the ordinates growth energy of the tissues in the various hosts. We find then that embryonic and adult invertebrate tissues and the embryonic tissues of lower vertebrates from one class (Curve I). This, however, does not necessarily imply that all these tisues behave in an identical manner, but that there exist some essential similarities. Our data are as yet by no means complete in this respect.

Very similar to this curve of the primitive tissues is that representing the growth of the transplantable tumors (Curve II).



Curve II. Transplantable tumors.

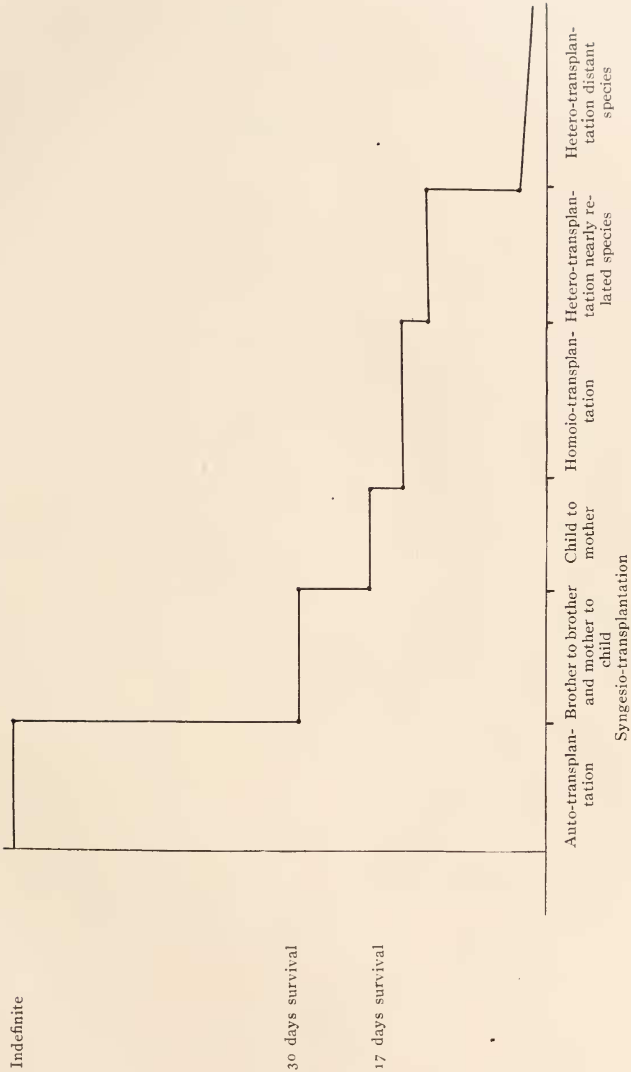
The latter, however, differ from the former in their sensitiveness to strain differences within the same species. In addition there may furthermore perhaps be found some differences in the behavior towards heterotoxins of invertebrate and embryonic tissues on the one hand and of transplantable tumors on the other hand.

From these curves differs very markedly the curve of the adult tissue of the higher vertebrates and similar to this is the curve of the large majority of the tumors, namely of those which generally are not transplantable, although in a limited number of individuals of the same species they may perhaps grow (Curve III.). The adult tissue of amphibia and fishes represents a transitional condition between type I. or II. and III.

Cellular and Psychical Discernment of Individuality.

We have shown that the cells of our body are able to discern in a quantitatively graded manner not only the difference between their own kind, between the constituent parts of the same individual on the one hand, and the cells of other individuals of the same species, on the other hand, but that they are able even to recognize in a graded manner degrees of relationship between members of the same family. We found especially the lymphocytic reaction a quantitative indicator of this relationship. We must therefore conclude that there are graded biochemical differences within the same family which these individual cells discern, and to which they react. These reactions represent as far as we are aware, the finest biochemical reaction known at the present time and on the basis of these reactions we may in a tentative manner postulate a graded system of contact substances which regulate the interaction of various tissues.

To return in conclusion to the starting point of our discussion, namely, the usual meaning of individuality, we saw that in the main, it designates a social-psychical way of reaction. We are able to differentiate between individuals as a result of certain functions of our central nervous system. If we now inquire how far the development of this kind of individualization is parallel to the power of cells of higher vertebrates generally to discern individuality, we are handicapped by the lack of data as to the power of animals to discern not merely members of a species,



CURVE III. Transplantation in adult mammals and birds. The curve of the non-transplantable tumors is somewhat similar.

of a litter or mother and mate, but individuals of the same species as such. This problem does not seem so far to have been considered by students of animal behavior. At least inquiries which I made among some prominent investigators in this field, failed to provide any definite data which might be used in this connection. From my own observations I am very much in doubt as to the ability of such animals as the common rodents to discern individuality in the sense in which we defined it. There seems to be little doubt on the other hand that higher animals like dogs and horses have such an individuality discernment, at least to a certain extent.

It is then very probable that the mechanisms which permit the ordinary tissue cells to discern and to react towards individuality have developed much in advance of that mechanism of our nervous system which permits us to recognize individuals in a conscious manner. On the other hand after the latter faculty has once developed, it has reached in man a very much greater degree of refinement in individualization than that exhibited by the discernment of individuality on the part of cells in general.

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THE ECOLOGY AND LIFE-HISTORY OF AMPHIGONOPTERUS AURORA AND OF OTHER VIVIPAROUS PERCHES OF CALIFORNIA.

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INTRODUCTION.

As the life-history of these fishes is intimately correlated with their viviparity, it may be of interest and pertinence to consider first some of the main features of viviparity in fishes. Most fishes are characterized by the prolific production of ova that are fertilized in an almost fortuitous manner. Within the group, however, some form of protection of the eggs has become repeatedly evolved, in correlation with a decrease in the number of ova and with a less random fertilization. This protection is variously accomplished by one or both parents,—by the burying of the eggs in relatively safe situations; the construction of nests of gravel, plants or bubbles; the driving of predatory

enemies away from the eggs or young; the gestation of the young within the mouth or blood pouch; the enclosure of the eggs in a tough capsule, or finally by the actual development of the young within the oviduct or ovary of the mother.

The degree to which this viviparity has become perfected varies widely in the different groups of viviparous fishes. Some teleosts, such as the scorpænoid fishes, give birth to thousands of minute embryos, still nourished by a relatively large yolk-sac; while others bear only a few young, but fully developed and capable of self-support, almost immediately after birth, in the normal manner of adult fishes. In some of these, as the Pœciliidæ, the embryos are nourished by the yolk in the egg, and a meroblastic type of cleavage persists. In the Embiotocidæ or viviparous perches on the other hand, the yolk is greatly reduced in bulk, the cleavage approaches the holoblastic type (according to Eigenmann, 1894, etc.), and the embryos are profoundly modified structurally.

The viviparous perches (see Figs. 1 and 2) comprise a compact group, the family Embiotocidæ (and the suborder Holconoti) of the Acanthopterygii or spiny-rayed fishes. The group is relatively old, apparently, for the many structural features correlated with viviparity are common to all of the species, and hence became fixed before the extensive generic differentiation characteristic of the family arose. Almost all of the species are generically distinct from the others, another situation suggesting the age of the group (cf. Eigenmann and Ulrey, 1894; Jordan and Evermann, 1898, and Hubbs, 1918). The immediate relationships of the Embiotocidæ not being apparent, nothing definite can be said concerning the origin of their viviparity.

The viviparity of the embiotocids was first definitely made known by Dr. A. C. Jackson in 1853, in a letter to the elder Agassiz. These fishes then almost immediately attracted the attention and study of a number of zoologists, among whom may be mentioned both Louis and Alexander Agassiz, Gibbons, and Girard. Later Ryder, and particularly Eigenmann, studied their embryology, and Jordan and Gilbert, Eigenmann and Ulrey and others also studied the group (see bibliography). I have re-

cently reviewed the family from a taxonomic standpoint (Hubbs, 1918), and have studied the life-history of several species, that of *Amphigonopterus aurora* (Fig. 2) in greatest detail. Although the following account is largely based on this species, comparisons with others are made in several connections.

Ecology.

Nothing whatever has been written concerning the life-history of *Amphigonopterus aurora,* the only species of the genus, and all that has been printed concerning its environmental relations is the statement that it is an inhabitant of the tide-pools of Monterey Bay, California, and that it feeds on algæ. Its habit of feeding

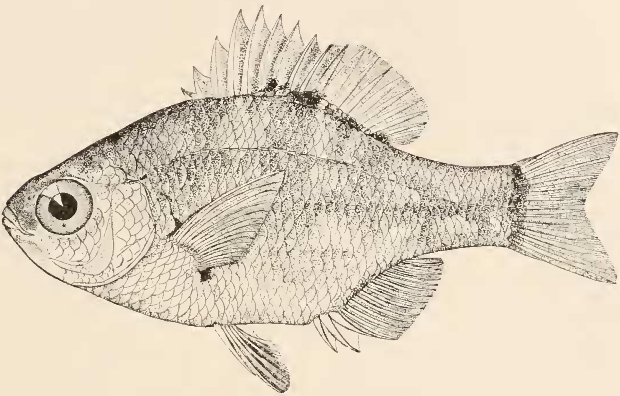

Fig. 1. Adult female of *Micrometrus minimus.*

on algæ, except when very young, when I found it feeding on copepods, is correlated with its tricuspid teeth and comparatively elongate intestine. Similarly I found that the related *Micrometrus minimus* (Fig. 1), though chiefly herbivorous, feeds on small crustaceans when young (Pt. Loma; December 31), and occasionally is caught on clam bait when adult. These two species alone comprise a distinct subfamily, the Micrometrinæ, which I have recently distinguished (Hubbs, 1918).

Amphigonopterus aurora inhabits the tidal pools and channels

along the rock-bound portions of the central California coast, its habitat differing widely from that usual to the species of the family, most of which live in the surf along sandy beaches, or in sheltered bays. In the preference of this species for this extreme type of habitat it is perhaps most nearly approached by *Micrometrus minimus*. The associational distribution of even these two species is, however, imperfectly complementary. Both range from the region of San Francisco southward in the cold coastal waters of central California to the reefs about Point Conception, where the habitat of *A. aurora* is abruptly terminated, whereas that of *M. minimus* is continued southward in the relatively warm waters along the coasts of southern and of Lower California. *Micrometrus minimus* is in fact most abundant in the warmer southern portion of its range, although fairly common northward, where the ranges of the two species coincide. Here, however, *M. minimus* occupies to a large extent a biotic association different from that of its congener, but adjacent to it: it lives and breeds in or not far below the lowermost tide-levels, mostly in the low, deep, plant-filled pools of the reefs (but also in enclosed bays and *esteros*). *Amphigonopterus aurora,* in contrast, is restricted to the reefs, and while breeding at times even in the same pools with its relative, more commonly lives and breeds in the pools and channels of medium tidal height, particularly those that are largely open, free of eel-grass and algæ, and floored with sand. The breeding season of *Amphigonopterus*, moreover, appears to begin earlier than that of *Micrometrus* in central California (see following section). Both of these fishes were found associated in the lower outer rock-pools of the California reefs with the following other species of the family: *Embiotoca jacksoni, E. lateralis, Hypsurus caryi* and *Cymatogaster aggregatus.*

In these open pools the fishes of this species swim about freely in schools[1] at low-tide, occupying the mid-water stratum chiefly,

[1] That these schools of *A. aurora* remain intact for considerable periods of time appears probable from two sets of observations. A number of pools on the reefs just south of Piedras Blancas, and just south of Pt. Sal, by careful observation over a period of several days (during a single series of low-tides in each case), were found to contain many more individuals than any of the adjoining pools, and to contain schools of apparently the same individuals,

occasionally leaping clear of the water (a habit observed only at Pt. Purisima, California, on August 13). During high-tide, however, they must seek the protection of crevices in the sides of the pools, for otherwise they would be dashed about on the rocks by the pounding, churning surf as it breaks on the reefs. In correlation with its preference for pools clearer of vegetation, and with its habits of swimming about rather more freely, *Amphigonopterus aurora* is more extensively silvery than *Micrometrus minimus,* and lacks the dark color pattern characteristic of that species[2] (see Fig. 1). In this connection we should recall that practically all free-swimming or pelagic fishes are silvery and lack the dark markings usually developed in fishes which live among rocks or plants.

Like most reef-fishes examined, *Amphigonopterus aurora* is not heavily parasitized, a fact apparently correlated with the strength of the wave and tidal currents on the reefs. Occasionally, however, a slender lernæan copepod was found attached to the inner surface of the base of the pectoral fin, or to the anal fin near its base.

Breeding Season.

The breeding season of *Amphigonopterus aurora* is the summer, approximately synchronous with that of *Cymatogaster aggregatus,* which breeds in bays and estuaries. It begins shortly before the first of June, as is evident from the observations made on the reefs of Piedras Blancas, California, during the first week of that month. Most of the many females taken on that occasion contained young, relatively few of the largest being spent. Furthermore, all of the hundreds of young in the higher pools were approximately of the size at which they are born. The breeding

judging from the approximate number of the fishes of each size. In a number of pools fished during the summer and fall, the young of the year of each sex were so uniform in size that it seemed probable that they had remained in that pool together since their birth at some time during the breeding season.

[2] The most conspicuous color feature of *Amphigonopterus aurora* (the one on which its specific name was based) is the longitudinal band of golden or orange color, which is rarely obsolete (a row of blotches of similar color and position often is present in *M. minimus,* representing this longitudinal band of *A. aurora*). In young specimens the vertical fins are dusky with a reddish tinge, the spinous dorsal, and in the male the anterior portion of the anal fin, being darkest; the pectoral, nearly colorless.

season was found to be still at its height about the middle of July, but to have ended long before October 26.

The breeding season of *Micrometrus minimus* in central California apparently commences somewhat later than that of *Amphigonopterus aurora*. Of females taken in the same pool near Piedras Blancas on June 2, those of *Micrometrus* contained embryos from 4.6 to 18.7 mm. long, none ready for birth, whereas those of *Amphigonopterus* contained embryos 12 to 34 mm. long, the largest obviously ready for birth, being similar to numerous young found at the same locality. A single female of *Micrometrus* collected at Point Purisima, California, on August 14, was spent, but all of those taken during June still contained young. Young showing considerable growth since birth were taken in reef-pools of southern California early in July, suggesting that the breeding season occurs earlier in the warmer waters to the southward.

Sex-ratio in Adults, Young and Embryos.

In the breeding pools poisoned near Piedras Blancas, the females were found to be more numerous than the males, in the proportion of nearly two to one: of those taken (by poison) and sexed, 139 proved to be males; 264, females.[1] An unrecorded observation by Dr. C. H. Gilbert on *Cymatogaster aggregatus* may have some bearing on this point: he has observed a single male "herding about" several females. On the contrary I observed several small fishes of the same species, presumably males, accompanying a mating pair (Hubbs, 1917). However this may be, the numbers of the sexes of the young fishes were found to be approximately equal in two pools at different localities fished in the autumn, after the close of the breeding season: in these two pools, 83 young males and 82 young females were obtained. The 35 adult specimens of *Micrometrus minimus* obtained in a single pool near Piedras Blancas comprised 19 males and 16 females.

A more accurate sex-ratio can be obtained by determining the sex of a series of embryos, by means of the secondary differences in the anal fin, which become clearly evident very early in the

[1] This difference in the sex-ratio may be determined by the greater longevity of the females.

development of *Amphigonopterus* and *Micrometrus* (see next section and Fig. 2). Of 630 embryos of *Amphigonopterus aurora* (from mothers one to four years old), 337 were found to be males, 293 females (sex-ratio: 100 males to 87 females); the ratio does not vary with the age of the parent fishes, being 100 to 86.5 in the embryos from the yearling females only. Of 150 embryos of *Micrometrus minimus* examined, 76 were males, 74 females; the proportion of males to females in each of the seven cases included was, 7 to 9, 9 to 14, 10 to 12, 10 to 13, 11 to 8, 13 to 12, 16 to 6. No tendency toward uniformity in sex even of embryos lying within the same ovarian sheets was evident; hence

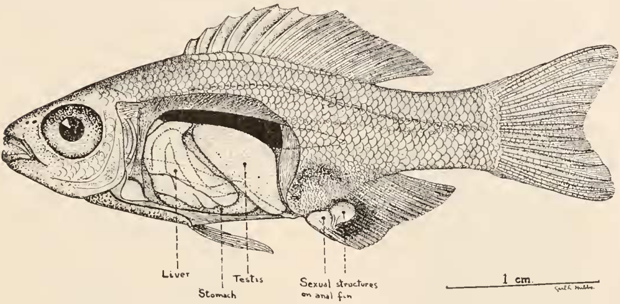

FIG. 2. Newly-born young male of *Amphigonopterus aurora,* from near Piedras Blancas, Cal. Dissected to show mature development of testes.

polyembryony does not occur. Eigenmann recorded similar data for *Cymatogaster aggregatus,* having distinguished the sexes cytologically.

Early Differentiation of the Sexes, and the Natal Maturity of the Males.

Secondary sexual differentiation is early manifest in the development of *Amphigonopterus aurora* and *Micrometrus minimus.* The differential number of anal rays characteristic of the sexes of each of these two species (Hubbs, 1918) is clearly apparent in embryos only 12 mm. long (the anal rays are first formed when a total length of about 10 mm. has been attained). Eigenmann was unable to distinguish the sexes in *Cymatogaster*

cytologically at earlier stages than this, although he traced the development of the sex-cells from much smaller embryos.

At the 15 mm. stage the sexual differences in the form of the anal fin are also apparent in *Amphigonopterus* and *Micrometrus,* although the gonad remains merely a fine strand of tissue; but at the 20 mm. stage the testes have begun to enlarge, and a slight thickening of the radial membranes marks the position of the

Fig. 3. Section of testis of a newly-born *Amphigonopterus*, showing several stages in spermatogenesis.

elaborate gland developed later on the anal fin of the male. The development of these primary and secondary sexual structures thence rapidly proceeds in *Amphigonopterus aurora* until birth, at which time the gland on the anal fin is fully elaborated (see Fig. 2), and all stages in spermatogenesis from the primordial germ cells to transforming spermatids at least are evident in the testis, the spermatogonia predominating. Just after birth, the

transforming spermatids and spermatozoa appear most abundant (Fig. 3): the testes, as well as the gland on the anal fin, are as well developed in these newly born males, as in the one-, two- and four-year-old males obtained during the breeding season. Both the primary and secondary sexual structures become greatly reduced in size during the autumn, winter and spring of the first, as of the succeeding years. In fact two young males only a few millimeters longer than the birth stage, collected in June, were already "spent." The writer has further determined that the testis becomes similarly enlarged in *Micrometrus minimus* and in *Cymatogaster aggregatus* just before birth. No evidence was obtained, however, to indicate that the males of *Embiotoca lateralis* are mature at birth; even the two one-year-old males of this species (111 and 116 mm. long to caudal) obtained near Pt. Sal, in California, on June 17, with breeding females, were immature. It is quite possible that the natal maturity of the males is confined to the smaller species of the Embiotocidæ.

This natal maturity of the males is particularly significant in view of the fact that the females bear young first at the age of one year. This phenomenon, while unique in the whole class of fishes, so far as the writer is aware, finds a physiological parallel in protandric hermaphroditism, in the frequent early maturing of the male (the "grilse") in the Salmonidæ, and in the earlier seasonal activities of the males of many animals.

Copulation, and the Storage of Spermatozoa.

Dr. Eigenmann (1894, p. 420) summarized one phase of his studies of another viviparous perch with this statement: "*Copulation takes place in Cymatogaster* during June or early July, although the eggs are not fertilized till the following December." He based this conclusion firstly on observations on the seasonal activities of the two sexes, and on the seasonal development of the testes in the male, and secondly, on the discovery of the presence of spermatozoa in the oviduct and in the ovarian folds of the female, during the latter part of the summer, and the autumn. The writer has been able to extend this evidence by the first observation of the copulation of this, in fact of any, embiotocid. The female of the pair in question upon capture was

found to contain only one young, partially protruding from the oviduct, and of the same size as numerous others recently born, found swimming about in the same body of water (Hubbs, 1917).

In *Amphigonopterus aurora* also it is probable that, copulation having taken place during the breeding season in the summer, the spermatozoa are retained in the females until winter (or possibly late autumn or early spring), when fertilization occurs and whence intramaternal development proceeds for several months; and that, therefore, *one year elapses between the time of copulation and the birth of the young.* Six lines of evidence lead to, or are at least not inconsistent with, these conclusions.

1. This condition apparently holds in *Cymatogaster aggregatus*, a distantly related species in which the breeding season is approximately synchronous with that of the present species, the structures correlated with viviparity similar, and in which the adults, and the young at birth, are of similar size to those of *Amphigonopterus aurora*.

2. Females taken in the autumn contained no young, and males secured on October 26, November 25 and April 1 had small and obviously non-functional testes, whereas all of the males taken with the breeding females in the summer had mature testes. This is true also of *Micrometrus minimus,* and perhaps of all embiotocids.

3. The smallest embryos, only 12 mm. long to the caudal fin, taken from any of the females secured in June, would presumably have attained their full embryonic size (about 30 to 35 mm.) late in the summer, toward the end of the breeding season. This fact suggests a moderately long period of gestation; the largest females, which at this time were bearing young, presumably had contained embryos for several months. A similar situation holds also in the case of *Micrometrus minimus*.

4. The largest females, which produce young early in the season, were found to be in a spent condition, not containing a new lot of embryos, during the months of July and August. Although the data are less complete, this condition appears to hold also in the case of *Micrometrus*.

5. The fact that the smaller yearling females bear fewer young

than do the larger ones (as also in *Micrometrus minimus* and other embiotocids) suggests that fertilization is delayed for a considerable period subsequent to copulation. Now it is a well-known fact, in oviparous as well as in viviparous fishes, that the larger females of a given species are more prolific than smaller ones, and that is the situation in this family. In the present instance, however, it is presumed that the copulation preceding the development of the first brood of young (those carried by the yearling females under discussion) takes place soon after birth, when there is little variation in size or age (see preceding section). If fertilization were then immediately effected, some rather anomalous method of fertilization, or of egg production or resorption, would have to be postulated to explain why those females which would be smaller at the end of pregnancy bear fewer young than those females, which for some reason, early birth or otherwise, are destined to be larger when their young are ready for birth. But if it be assumed that fertilization is delayed for some time, until a considerable variation in size shall have arisen, the bearing of the fewer young by the smaller yearling fishes becomes no longer such a special problem.

6. Finally, the young males are sexually mature immediately after birth (see preceding section), at a time when they are associated only with the newly-born females, which apparently do not bear young until the next breeding season, one year later. A similar situation presumably holds in the cases of *Micrometrus minimus* and *Cymatogaster aggregatus*.

Embryonic Development and Natal Metamorphosis.

The developing embryos of the Embiotocidæ, in compensation for the reduced amount of yolk in the relatively minute egg, derive their nourishment almost entirely from the nutritive ovarian fluid in which they are bathed. This fluid, as Eigenmann (1894, etc.) determined, is circulated by the action of cilia through the embryos. The portion of the alimentary canal in which absorption chiefly takes place is doubtless the hypertrophied hind-gut, which in the embryos of *Amphigonopterus* and *Micrometrus*, as of other genera of the family, is a wide but thin-walled tube nearly filled with long, hollow, vascular villi. The respiration of

these embryos is seemingly largely effected over the surface of the body and fins, especially in the highly elevated vertical fins, in the distal dermal flaps of which the large interradial vessels form an extensive capillary net-work (cf. Ryder, 1885, 1893). The embryos, thus supplied with food and oxygen, pass through their development in the ovary, lying tightly packed against the ovarian walls and ovarian sheets, some directed forward, others backward, in such a fashion, as Agassiz long ago pointed out, as greatly to conserve space.

About the time of birth, the young of *Amphigonopterus* (and of other embiotocids) undergo a notable change, which may be termed the natal metamorphosis. The body becomes thicker, the flesh firmer; the vertical fins become shorter and less flexible, the interradial vessels smaller, the dermal flaps obsolete, and the hind gut more nearly normal in structure. The scales have already developed so far that they are widely imbricate, and the chromatophores have been formed in large numbers, but the body even in the largest embryos is very much paler in color than in the newly born young, particularly the males, which are even darker than the adults. In other species, as *Embiotoca lateralis*, and *Hypsurus caryi*, the latter as described by Agassiz, a sharply defined color pattern is developed before birth. The viviparous perches thus lose nearly all traces of embryonic peculiarities immediately before and after birth.

The young of *Amphigonopterus aurora* at birth vary in length approximately from 30 to 35 mm. (the caudal fin excluded), being about one third or one fourth as long as their mothers, as in other species of the family. Among several hundred examined early in June, the smallest free-swimming young was 29.0 mm. long, the longest unborn embryo, 35.5 mm. In a given series of embryos from one female, the variation in length is seldom more than one or two millimeters; thus the two sexes in *Amphigonopterus* are seen to be of at least approximately the same size at the time of birth; the differential rate of growth is wholly, or almost wholly, postnatal.

A female of *Embiotoca lateralis*, 257 mm. long to caudal, caught near Piedras Blancas, California, on June 2, contained 26 young 46 to 49 mm. long, not quite ready for birth. A slightly

larger female, 265 mm. long, collected in the same pool, also contained 26 young, but these were larger, 50 to 54 mm. long, and similar to recently born young obtained near Piedras Blancas, Pt. Sal and Pt. Arguello. Another female of this species, 200 mm. long and 125 mm. deep (exclusive of fins) contained only ten young, 55 to 58 mm. long, some having apparently been already born. The newly born young obtained, all during June, varied in standard length from 43 to 58 mm. A variation in size at birth of at least 16 mm. is thus suggested. Possibly, however, a slight decrease in actual length accompanies the metamorphosis of this species (as in *Albula vulpes* and the eels).

In the case of *Micrometrus minimus,* the extreme lengths of the embryos of a single female were found to differ normally from 0.0 to 3.0 mm. In three cases, however, the variation was much greater: in one lot of six embryos, the individual lengths were 5.5, 9.5, 10.8, 11.5, 14.3, and 14.7 mm.; in a second lot, five were 6.6 to 7.6 mm. long, a sixth, 2.7 mm.; in the third case, all but one of the fœtuses were 12.0 to 13.7 mm. long, the abnormal one being 9.0 mm. long, and provided with a strongly sigmoid vertebral column and a single eye, represented only by a mass of black pigment. Occasionally, a male embryo was found to be slightly larger or smaller than any of its fellows. If the 16 embryos in one female, 7 were males 16.3 to 18.7 mm. long, while 9 were females 17.0 to 18.0 mm. long; the average as well as the mean length for each sex was 17.5 mm. In another lot of 23 fœtuses from one female, 10 were males 20.0 to 22.3 mm. long (average length, 21.8 mm.), while 13 were females 20.4 to 23.0 mm. long (average length, 21.7 mm.).

Soon after birth the young of *Amphigonopterus aurora* leave the lower pools in which they were born, only a few remaining, probably for a very short time, in company with the breeding adults. They make their way thence into the pools accessible only at high tide, in such abundance that these pools, which are usually of small size and shallow, not infrequently harbor astonishingly large numbers of these young fishes. Such pools provide a large degree of seclusion from predatory enemies, as well as the warmest available water, in which the rapid growth of the first months may take place. This concentration and segregation

of the young may also be correlated with selective mating, especially in view of the natal maturity of the males.

Period of Breeding of Females of Different Size and Age.

The following table gives the average length of the young found in each of fifty one- and three-year-old[1] females of *Amphigonopterus,* all of which were obtained near Piedras Blancas, California, during the first week of June, 1916. Both adults and embryos were measured when freshly caught, prior to their preservation. The smallest young are not nearly developed to the stage at which they are born: it is improbable that the smaller females give little to young notably smaller than those of the larger females.

Table I.

Size of Young of Amphigonopterus aurora Carried by Females of Different Length and Age.

Average Length of Young (in mm. to Base of Caudal)	Lengths of Females Carrying Young of Foregoing Length.	Age of Females (End of Given Year since Birth).
12	77	I. (1)
13	96	I. (1)
14	85	I. (1)
15	84	I. (1)
16	76, 77, 82, 85	I. (4)
17	77, 86, 90, 95	I. (4)
18	78, 81, 84, 85, 88, 94	I. (6)
19	81, 87, 92	I. (3)
20	88, 88	I. (2)
21	92, 94, 95, 98, 98, 99	I. (6)
22	85, 87, 91	I. (3)
23	—	——
24	102; 122	I. (1); III. (1)
25	95, 103	I. (2)
26	103	I. (1)
27	102	I. (1)
28	95, 96; 118	I. (2); III. (1)
29	112	III. (1)
30	—	——
31	126	III. (1)
32	121, 123, 128	III. (3)
33	122, 123, 128	III. (3)
34	129	III. (1)

[1] The method of age-determination by scale examination will be discussed later.

The foregoing table indicates clearly—and the evidence has been confirmed by the writer by a study of material obtained at other localities—that *the smaller and younger females of Amphigonopterus give birth to their young later in the season than do the larger and older females.* This is true likewise of *Micrometrus minimus* (Table II.), and as Eigenmann (1894) has demonstrated, of *Cymatogaster* and other genera of the family (but Eigenmann made no definite age-determinations). This delayed breeding of the smaller, younger females of *Amphigonopterus* and other Embiotocids may be an advantageous adaptation, allowing the growth of the yearling females to be continued, as the structure of the scales indicates it does, during the breeding of the older females. Most of the females being one-year fishes in *Amphigonopterus* at least, this added growth would seem to admit of a material increase in the number of young produced.

Number of Young Born by Females of Different Size and Age.

The following table III., based upon data obtained from 48 breeding females of *Amphigonopterus aurora* (all obtained near Piedras Blancas during the first week of June, 1916), conclusively shows that *the smaller females bear fewer young than do the older and larger ones,*—

the one-year-old females with 5-9 young being 76–94 mm. long (average length, 83 mm.);

the one-year-old females with 10–15 young being 85–103 mm. long (average length, 96 mm.);

the 3- or 4-year-old females with 16–30 young being 121–129 mm. long (average length, 125 mm.).

Exceptional cases, excluded from this summary, are those of a three-year female with but 9 small young, and a one-year female with 19 young. Dr. Eigenmann (1894) has similarly found that the smaller females of several other species of the Embiotocidæ bear fewer young than do the larger ones, and the data presented in Table II. shows that this holds true in the case of *Micrometrus minimus.*

Table II.

Number and Size of Embryos of Micrometrus minimus Carried by Females of Given Age and Size (in Mm. to Caudal Fin).

Locality (Approximate).	Date.	Mother.		Embryos.	
		Age.	Size.[1]	Number.	Size.[1]
Monterey........	Mar. 26–Apr. 2	I.	59	7	3.7–4.3
"	"	I.	61	7	5.0–5.5
"	"	I.	65	7	5.0–6.0
"	"	I.	67	10	6.0–6.6
"	"	I.	71	9	4.6–5.4
"	"	I.	73	10	7.0
"	"	I.	74	10	7.6–8.3
"	"	II.	86	13	8.6–10.6
"	"	II.	92	17	9.3–10.2
"	"	II.	92	17	11.6–13.0
"	"	II.	92	19	11.6–12.7
"	"	III.?[2]	106	22	16.6–17.6
"	"	"	107	23	16.4–17.3
"	"	"	110	24	18.0–21.0
"	"	"	113	25	18.6–21.6
"	"	"	116	19	21.6–23.5
Avila............	May 25	III.?[2]	110	21	21.7–23.6
"	"	VI.?[2]	198	....	....
Piedras Blancas...	June 2	I.	58	3	5.3–5.5
" " ...	"	I.	60	2	4.6
" " ...	"	I.	60	4	6.7
" " ...	"	I.	61	6	10.0–10.4
" " ...	"	I.	66	8	7.4–8.9
" " ...	"	I.	67	7	10.0–11.8
" " ...	"	I.	70	6	5.5–14.7
" " ...	"	I.	70	8	12.7–13.4
" " ...	"	I.	71	9	12.3–13.0
" " ...	"	I.	73	8	12.2–12.5
" " ...	"	I.	74	8	15.0
" " ...	"	I.	76	12	9.0–13.7
" " ...	"	I.	79	10	16.0–18.0
" " ...	"	I.	79	13	15.7–17.7
" " ...	"	II.	85	15	15.0–17.0
" " ...	"	III.	97	16	16.3–18.7
Pt. Sal...........	June 17	I.	69	6	11.0–11.6
"	"	I.	72	6	2.7–7.6
"	"	I.	74	7	13.2–13.7
"	"	III.?[2]	96	17	17.0–18.3
"	"	V.?[2]	114	23	20.0–23.0
"	"	VI.?[2]	129	22	....
Pt. Purisima.....	June 18	I.	64	2	10.0
"	"	I.	67	6	8.3–9.3
"	"	I.	76	6	13.4–15.0

[1] Preserved material measured.

[2] Age uncertain, owing to the development of apparently accessory annuli.

Table III.

Size of Young Carried by Females of Different Size and Age.

Number of Young.	Lengths of Females Carrying Young.	Age of Females (End of Given Year since Birth).
5	87	I. (1)
6	76, 82	I. (2)
7	77, 77, 78, 79, 81, 84, 85, 87, 88	I. (9)
8	77, 81, 90	I. (3)
9	84, 85, 86, 88, 94; 122	I. (5); III. (1)
10	92, 96, 103	I. (3)
11	85, 88, 91, 95, 95	I. (5)
12	92	I. (1)
13	98, 98, 102	I. (3)
14	94, 95, 102	I. (3)
15	99, 103	I. (2)
16	129	IV. (1)
17	—	——
18	122	IV. (1)
19	96; 123	I. (1); III. (1)
20	122, 128	III. (2)
21	—	——
22	121, 128	III. (2)
23	123	IV. (1)
24 to 29	—	——
30	126	III. (1)

Seasonal Marks (or Annuli) on the Scales.

During recent years there have been conducted numerous studies, of biological interest and economic significance, based upon age-determinations and the computed rate of growth of fishes. In these studies there has been developed and rather thoroughly tested a method of age-determination involving an interpretation of the seasonal rings indicated in scales, otoliths and certain bones; the scales have been most widely used. It has been demonstrated, most definitely in the salmonoid fishes, that the circuli covering the surface of the scales (cf. Fig. 4) become weaker in structure, more interrupted and more closely approximated during each winter, apparently as a result of the lessened physiological activity and retarded growth of the fish at that season. In certain fishes which have been investigated, these winter marks or *annuli* are indicated not so much by an approximation of the circuli as by a change in their direction and an interruption in

their course, along a line parallel with the scale margin (cf. particularly Taylor, 1914).[1] The reason for the formation of this type of annulus is the fact that the circuli toward the end of each year's growth gradually become more strongly curved, whereas those marking the new growth are straighter. This is the type of annulus formed on the scales of the Embiotocidæ (see Fig. 4),

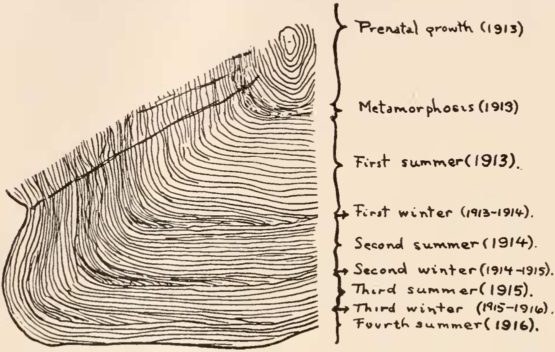

FIG. 4. Lateral field of a scale from a three-year-old female of *Amphigonopterus aurora,* showing the annuli.

although an approximation of the circuli is also frequently evident, especially on the basal and lateral fields.

That the annuli on the scales of *Amphigonopterus aurora* are formed during the winter is evident from a consideration of the following facts. A series of young from New Monterey collected on October 26, and another lot from near Pillar Pt., California, obtained on November 25, show no trace of an annulus at the margin of their scales, and had not yet attained the computed length at which this mark had been formed in larger specimens. Excluding recently born young, the smallest examples of either sex among those taken near Piedras Blancas during the first week of June, and also the young specimens secured near Pillar Pt. on April 1, have a single annulus on their scales, some dis-

[1] The statement by Taylor that no approximation of the circuli occurs in the annuli is partially erroneous (particularly as it applies to salmonoid fishes), as is also his conclusion that the annuli of the fishes which he studied were formed during the summer (even Taylor's own data indicates the contrary).

tance within the margin. Thus it appears that considerable growth had taken place since the annulus was formed. The actual amount of this growth (determined by a method discussed later), in 50 of the specimens from near Piedras Blancas, was estimated to have varied from 10 to 28 mm.; in more than half (28) of these the growth had been 14 to 18 mm., while the average growth computed to have occurred between the formation of the first two annuli of older fishes from the same locality, was about 24 mm. No annulus was found on the margin of the scales of gravid nor recently spent females, indicating that it is not a breeding mark.

In certain other species of the family, the annuli are doubled in a confusing fashion, suggesting the possibility that two annual checks in growth are registered on the scales, one during the winter and the other during the breeding season. For instance, the scales of a 200 mm. female of *Embiotoca lateralis,* taken on June 17, when bearing young, show five typical winter annuli, and in addition to these, and located between them, less distinct but similarly formed rings, the outermost at the extreme margin of the scales. Similarly in *Micrometrus minimus* the annuli are often closely approximated or doubled (beyond the second winter annulus); in these cases also the outermost annulus is located at the margin of the scales of females carrying young. Such a condition is seldom apparent in *Amphigonopterus,* but may have introduced an occasional error in the interpretation of the scales of the fishes three or four years old.

The annuli or seasonal rings on the scales of *Micrometrus minimus* closely resemble those of *Amphigonopterus aurora* (except in the more frequent appearance of doubled annuli, as just noted). The outermost annulus is located at some distance within the margin of the scales of yearling specimens taken in late spring and early summer in central California. In several specimens of both sexes, young of the preceding summer, taken at Pt. Loma on December 31, the single winter annulus is on or immediately within, in one male considerably within, the margin of the scale. These facts indicate that the annuli of *Micrometrus* are winter marks, that the first is formed in December in southern

California, and that there is some variation in the time of their formation.

Metamorphic Annulus.

The scales of even the largest embryos of *Amphigonopterus aurora* and of *Micrometrus minimus* are marked from focus to border by evenly spaced, concentric striæ; those of all but the most recently born young, on the other hand, are marked near the margin by a zone in which the circuli are finer and more closely approximated than on either side, and frequently angulated, their course on the scale within this zone being slightly different from that without. This mark, which is formed during the summer, resembles the winter checks or annuli formed farther out on the scales of older fishes, and perhaps quite as closely simulates the annulus on the scales of the Pacific salmon. As a distinctive name, the term *metamorphic annulus* is proposed for this mark. It is likewise indicated on the scales of *Cymatogaster aggregatus* and of other species of the family; the time of its formation (as indicated above soon after birth during the summer) has been confirmed in the case of *Embiotoca lateralis*.

The cause leading to the formation of the natal annulus is apparently a temporary retarding of growth immediately after birth, just as the other annuli are supposed to be formed as a consequence of the decreased nutrition and growth of the fish during the cold season. In this connection there should be recalled the sudden alteration of the method of feeding and respiration forced upon the young of these fishes at birth. They are then cast out into a very different medium, from which oxygen must be absorbed mostly through the gills, rather than through the skin and the tips of the fins. Instead of merely passing through themselves the nutritive fluid with which they were surrounded, they must now feed in the normal fashion of fishes. It is obviously these changes in the manner of living, which stamp a lasting mark on the scale. The metamorphic annulus significantly is usually more sharply evident in the males than in the females of those embiotocids known to be characterized by the natal maturity of the males.

Comparative Size of the Sexes at Different Ages.

Scales were retained from numerous specimens of *Amphigonopterus aurora* of measured length, all obtained near Piedras Blancas, California, on June 2 and 4, 1916. Excepting a few selected to represent extreme sizes, these were chosen at random from the large number collected. The annuli, discussed above, are well developed on these scales, their number indicating the approximate age of the fish (the time of intramaternal development, about one half year, being arbitrarily excluded). Thus the presence of a single annulus, in addition of course to the natal annulus, indicates that the fish was born in the preceding summer, and that it is just completing or has just completed, the first year

Table IV.

Length to Caudal Base of Females of Amphigonopterus aurora of Different Ages.

Age, One Year.		Age, Three Years.		Age, Four Years		Age, Five Years.		Age, Six Years.	
76 mm.	(1)	116 mm.	(1)	122 mm.	(1)	141 mm.	(1)	138 mm.	(1)
77	(3)	117	...	123	(1)				
78	...	118	(2)	129	(2)				
79	(1)	119	(1)	136	(1)				
80	...	120	(1)						
81	(3)	121	(1)						
82	(1)	122	(2)						
83	...	123	(1)						
84	(3)	124	...						
85	(4)	125	(3)						
86	(1)	126	(1)						
87	(2)	127	...						
88	(3)	128	(2)						
89	(1)								
90	(2)								
91	(1)								
92	(3)								
93	(1)								
94	(2)								
95	(4)								
96	(2)								
97	(1)								
98	(1)								
99	(2)								
100	(2)								
101	...								
102	(2)								
103	(2)								
104	...								
105	(1)								
106	...								
107	...								
108	(1)								

of its free-swimming life. As these fishes were obtained in the summer of 1916, those with three annuli were born in the summer of 1913, etc.

The collecting done near Piedras Blancas and at other localities indicated that the males average decidedly smaller than the females. It is of interest to have the field observations definitely confirmed by age-determinations.

Table V.

Length of Males of Different Ages.

One Year.	Two Years.	Four Years.
59 mm. (1)	82 mm. (1)	89 mm. (1)
60 (1)		
61 (1)		
62 (1)		
63 (1)		
64 (3)		
65 (4)		
66 ——		
67 ——		
68 (4)		
69 (2)		
70 (2)		
71 (1)		
72 (3)		
73 (1)		

The wide difference in the size of the two sexes of *Micrometrus minimus* as well as of *Amphigonopterus aurora* (Table VI.) appears to be of particular significance in the case of a small viviparous fish. The female of *Amphigonopterus* carries from 5 to 30 young which attain before birth more than one fourth the length of their mother, whereas the testes of the male are relatively small for a fish,—a fact determined by the conservation of spermatozoa, correlated with copulation (similar size relations prevail in certain other and probably in all embiotocids, and in many of the viviparous pœciliids). The differential rate of growth producing the relatively smaller size of the adult males is entirely or almost entirely postnatal, as previously indicated; in this connection it should again be recalled that at birth, only the males are mature.

TABLE VI.

THE COMPARATIVE SIZE OF THE SEXES IN AMPHIGONOPTERUS AURORA AND MICROMETRUS MINIMUS.

Species.	Approximate Locality (California).	Date of Collection.	Age (Winters).	Length in Mm. to Caudal.	Sex.	Number of Specimens.
A. aurora....	Piedras Blancas	June 2–4	I.	59–73	♂	25
" "	" "	"	I.	76–108	♀	50
" "	" "	"	II.	82	♂	1
" "	" "	"	III.	116–128	♀	15
" "	" "	"	IV.	89	♂	1
" "	" "	"	IV.	122–136	♀	5
A. aurora....	Pacific Grove	....	II.	134–139	♀	2
A. aurora....	Pillar Pt.	Nov. 26	0	48–70	♂	18
" "	" "	"	0	41–56	♀	17
M. minimus .	Pt. Loma	Dec. 31	I.	51–52	♂	2
" " .	" "	"	I.	63–64	♀	2
M. minimus .	Piedras Blancas	June 2	I.	52–62	♂	15
" " .	" "	"	I.	58–79	♀	14
" " .	" "	"	II.	66	♂	1
" " .	" "	"	II.	85	♀	1
" " .	" "	"	III.	68–71	♂	3
" " .	" "	"	III.	97	♀	1
M. minimus .	Pt. Sal	June 17	I.	62	♂	1
" " .	" "	"	I.	69–74	♀	3

RATE OF GROWTH.

The rate of growth of a fish which develops seasonal marks on the scales can be readily computed with considerable accuracy from the known length of the fish at the time of capture and the measurements of one of its scales. The number of scales remaining constant throughout life, the scale increases in length along its horizontal axis in direct ratio to the increase in the length of the fish. Thus for a fish of given total length, the length attained at each winter, and consequently the growth increment of each successive year, may be computed by the use of the following formula:

$$\frac{\textit{length of fish at the time of capture}}{\textit{length of fish at winter } x} = \frac{\textit{length of scale to margin}}{\textit{length of scale to annulus formed in winter } x}$$

Several possible sources of error in the use of this formula are apparent. The scales do not develop until considerable growth has occurred. As the length of the scale is usually measured, for the purposes of growth computations, only from the focus cephalad to the basal margin, an unequal growth of the different fields of the scales may introduce an error. There is some variation in the time of formation of the annuli. The scales often overlap less widely in young fishes than in adults, rendering the computation of size for the first winter too small. Again, the length of the fish is measured, for growth computations, from the tip of the snout to the base of the caudal fin. There is thus included the length of the head, which becomes relatively shorter with increased size in most fishes. As the formula assumes that the head and body increase in the same ratio, the computed length for the young fish at a given stage would be accurate only if the head were then of the same proportional length. The head being relatively longer, however, the computation is too small. It is doubtless these factors (probably all of them) which have rendered the computed length of the young of *Amphigonopterus* at the time of the formation of the natal annulus (just after birth) to be less than the observed length at that stage (20 to 32 mm., instead of 29.0 to 35.5 mm.).

TABLE VII.

COMPUTED RATE OF GROWTH OF FEMALES OF AMPHIGONOPTERUS AURORA.[1]

Period of Growth.	Growth.	Specimens
To formation of natal annulus	20–32 mm.	70
Thence to end of first winter	29–68 mm.	69
Between first and second winters	14–35 mm.	20
Between second and third winters	12–27 mm.	20
Between third and fourth winters	13–18 mm.	5
Between fourth and fifth winters	13 mm.	1
Between fifth and sixth winters	4 mm.	1

[1] Based upon material collected near Piedras Blancas during the first week of June, 1916.

It is evident from these figures, as well as from supplementary data obtained from collections made on other occasions, that *the growth of the first half year (between birth and the first winter) is greater than that of any subsequent whole year.* This estimate

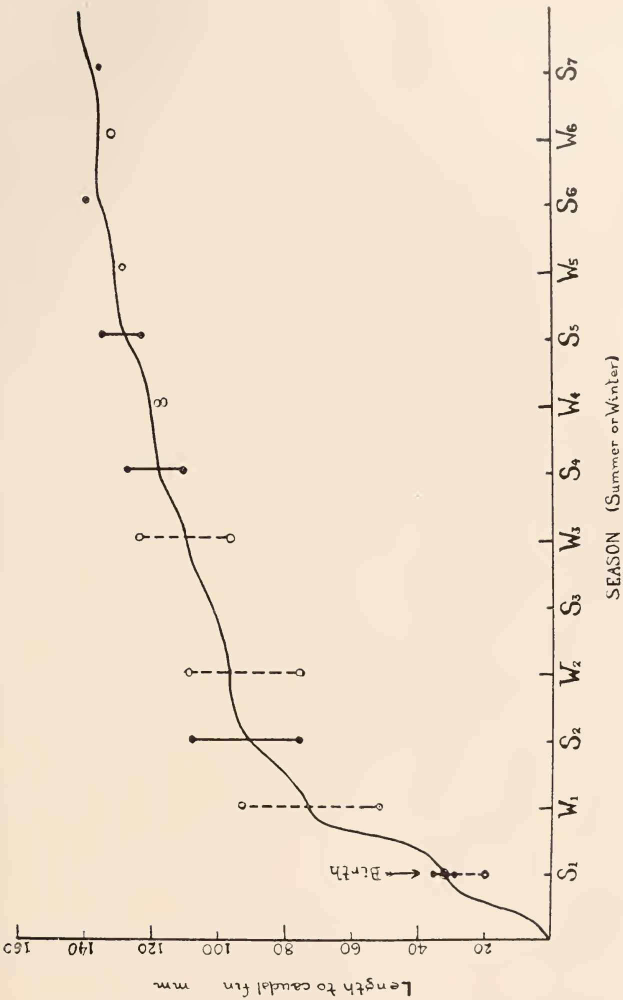

FIG. 5. Estimated average growth curve of females of *Amphigonopterus aurora* on the reefs of San Luis Obispo County, California. Lengths for summers obtained by measurement of specimens collected on June 2–4, 1916; lengths for winters obtained by computation from scale-measurements of the same fishes.

appears particularly significant, in view of the fact that the females become pregnant at or immediately after their first winter. As usual in fishes, their is no evidence that the growth ever wholly ceases during life, although it is markedly and increasingly retarded with age.

The growth of the single four-year-old male of *Amphigonopterus* obtained near Piedras Blancas was computed from the direct ratio of scale length to fish length. The length of the head and body to the end of the formation of each annulus was thus estimated to have been as indicated below.

Length at end of formation of natal annulus	26 mm.
" " " " " " first winter annulus	50 mm.
" " " " " " second winter annulus	63 mm.
" " " " " " third winter annulus	73 mm.
" " " " " " fourth winter annulus	84 mm.
" " " " fourth year (on June 4, 1916)	89 mm.

The growth of this male, although slower, was quite similar to that of the females, being greater between birth in the summer and the first winter, than during any subsequent whole year; the check in growth rate in this case follows the second, rather than as usual the first, period of breeding.

The writer has found but one published record of a direct observation on the rate of growth of a viviparous perch. It was made on aquarium fishes by the late Charles Frederick Holder, and published anonymously and without identification of species.[1] The obscure but pertinent passage is as follows. "The young, ten or twenty in number, born in the summer, are from an inch and a half long at birth, and attain half their adult size the first winter, and their full growth in about two and a half or three years." Dr. Eigenmann (1894) has remarked on the large size of the smaller breeding females of *Cymatogaster,* which he correctly assumed to be one year old. A similar rate of growth holds in the case of *Micrometrus minimus.*

In the course of his extensive investigations of the life-history of the sockeye salmon (*Oncorhynchus nerka*), Dr. C. H. Gilbert

[1] Another note by the same author makes it evident that the species observed was *Cymatogaster aggregatus* (see Bull. U. S. Bur. Fish., Vol. 28, 1908 (1910), p. 1139).

(1914, pp. 61–71) has induced an important generalization, *the law of growth compensation.* Those salmon which grow most in their first year (as a result of earliest hatching or of other causes), tend on the average to grow least in their succeeding years, while those which have attained a relatively small size at the end of the first year, grow with accelerated speed during the next years. The physiological mechanism of the salmon appears to regulate its growth in such a fashion that the length of the adult fishes of each race varies but little. It was hoped that it might be determined whether this law of growth applies to *Amphigonopterus,* but so few fishes more than one year of age were examined, that the data are incomplete. The evidence being suggestive, however, is presented in the following table (based upon the material from Piedras Blancas).

TABLE VIII.

COMPUTED LENGTHS OF TWENTY 3- TO 6-YEAR-OLD FEMALES OF AMPHIGONOPTERUS AT THE END OF FIRST THREE WINTERS.

	Length at End of First Winter.	Length at End of Second Winter.	Length at End of Third Winter.
	52	76	97
	58	91	108
	61	94	109
	62	93	107
	62	96	121
	63	98	110
	64	80	100
	67	101	115
	67	94	113
	68	82	109
	72	93	113
	74	101	120
	75	103	119
	77	101	120
	79	98	124
	80	108	120
	81	96	118
	83	103	120
	85	99	111
	93	109	124
Variation	52 to 93	76 to 109	97 to 124
Range	41	33	27
$\frac{\text{Range}}{\text{Mean}}$	.57	.40	.26

It appears probable that the variation in size of *Amphigonopterus* at the end of the third winter is less than at the end of the

first winter; that the principle of growth compensation is applicable to this embiotocid. Similar data gathered from one-year-old females strengthen this conclusion.

TABLE IX.

COMPUTED SPRING GROWTH OF SPECIMENS WHICH HAD ATTAINED EITHER A SMALL OR A LARGE SIZE AT THEIR FIRST WINTER.

Length at End of Formation of First Annulus.	Spring Growth (in Millimeters).																		
	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
Less than 75 mm.	–	–	1	–	3	1	2	5	3	–	2	4	2	1	1	–	–	–	1
More than 75 mm.	1	1	2	2	1	6	1	3	2	2	1	–	–	–	1	–	–	–	–

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STUDIES OF THE BIOLOGY OF FRESHWATER MUSSELS.[1]

Experimental Studies of the Food Relations of Certain Unionidæ.

William Ray Allen.

CONTENTS.

1. Introduction.

The writer, while a member of the Indiana University Biological Station at Winona Lake, Indiana, devoted several summers to ecological studies of the freshwater mussels. The work was done under the direction of Dr. Will Scott, and formed a part of his general limnological program. A first paper on the feeding habits of the Unionidæ appeared in 1914. The present paper has expanded the former through the use of experimental

[1] Contribution from the Zoölogical Laboratory of Indiana University No. 174; submitted as a partial fulfillment of the requirements for the degree Doctor of Philosophy.

methods, and approaches the study of the crystalline style from the physiological standpoint. In subsequent papers I hope to present further studies along the lines of reactions to stimuli and distribution.

For the study of the smaller glacial lakes and their inhabitants the region is especially favorable. Winona Lake has a maximum length of two miles, and being of the kettle-hole type, is deep. Its greatest depth is eighty feet. The bivalve population is limited to a shelf about its margin. Three small creeks drain into the lake. Their course follows the flat, marshy land between moraines, and their volume is small and comparatively constant. The Unionids have not extended up into the creeks. The lake forms the upper limit of their distribution in the Winona drainage system.

Eight species of mussels have been recorded from this lake: *Lampsilis luteolus, Anodonta grandis, A. edentula, Quadrula rubiginosa,* and *Lampsilis subrostratus* are common, the first two being very abundant. *Lampsilis glans, Micromya fabalis,* and *Margaritana marginata* are rare. I have found a single specimen of a ninth species, *Quadrula undulata.* All these mussels are of the "lake" type, as contrasted with "river" mussels.

For most of the following experimental studies *Lampsilis luteolus* and *Anodonta grandis* have been employed. For work during the winter, and for comparison with the above mentioned lacustrine forms, mussels from White River were used. These include *Quadrula heros, Q. pustulosa, Lampsilis anodontoides, L. ligamentinus,* etc. They were collected from the east fork of White River, near Shoals, Indiana, and from the west fork of the same river near, Gosport, Indiana.

2. Constancy of the Feeding Activity.

In a previous paper I stated that the lake mussel continues feeding at nearly all times, when under normal conditions (Allen, '14). Virtually all the observations made since the publication of that paper have been of a confirmatory nature. Rarely is a freshly collected mussel found to be without food material in its alimentary tract, often much of it wholly undigested. When

brought into captivity defecation usually continues to occur even after several hours, and indicates that feeding has at no time been long suspended. The normal position of the palps, gills, and mantle with respect to each other is favorable to ingestion. The structure of the mussel is such that it requires a greater effort to refrain from feeding than to continue feeding. The ciliary apparatus is in constant activity, regardless of the presence or absence of food. The gills are at all times siphoning water. Particles suspended in the water are at all times being sifted out and caught upon the mucous secretions of the gills. The gills have no way of rejecting such collections. They are always passed on by the fixed ciliary tracts of the gills to a given point on the lower margin of the inner gill which hangs between the labial palps. Without an adverse stimulus and an avoiding movement of the palps the collections pass on to their apposed surfaces. Thence it is but a short distance to the mouth.

If, despite the structure of the mechanism of ingestion, one might still doubt that feeding is a constant process, he is forced to grant that feeding is speedily resumed by mussels artificially starved. Those starved a sufficient length of time to get rid of the crystalline style, when placed again in lake or stream, begin the renewal of the style within fifteen to thirty minutes.

(a) *The Feeding Posture.*

To what extent feeding is conditioned by the position of the mantle, palps, and gills the following experiment will show:

Some *Lampsilis* and *Anodonta* were starved several days to insure the disappearance of the crystalline styles. The renewal of these organs was then taken as an index of the feeding activities. Various individuals were placed in the lake, in lake water, and in Pocahontas creek. Some were partially inserted in sand in the normal posture, others with the siphons turned upward, others laid upon the right or left side, and still others upon the hinge and having the gape of the shell uppermost.

At intervals some from each situation were examined. The styles were found to be reformed in all of them at about the same rate. The position of the ciliated parts, with relation to one

another and to gravity, was obviously of no consequence. Food could be passed from the gills to the palps as readily upward as downward or horizontally. Due to the buoyancy of the mucus in which food material is gathered and passed to the stomach, so near the specific gravity of the surrounding water, cilia need exert only a very slight traction upon the food masses to move them in any direction.

No Unionids have acquired an asymmetry like that of the oyster, but some species, particularly Quadrulæ, are always found lying upon one side or the other. They feed as readily upon one side as upon the other. Neither the right side nor the left is favored. There is no exceptional arrangement of ciliated parts within the mantle chamber for countering the unsymmetrical pull of gravity, unless the unusual size of the labial palps of some species may be interpreted as a means of preventing the loss of food between gills and palps.

No matter, then, in what position the gills and palps may be, food is readily passed mouthward, and the process of food collecting goes on constantly in the absence of adverse stimuli. Only with the closure of the siphons is the streaming of fresh material interrupted. Only in case of powerful stimulus are the palps caused to move out of line, away from the inner gill, thus refusing food masses entirely. It has been difficult to demonstrate the constancy of the food stream upon the contiguous faces of the labial palps. The ciliated furrows (p. 234) have the functions of accepting or rejecting material, but to what extent the latter function is exercised under normal circumstances it is difficult to determine directly. When fed in high concentrations certain specific substances were rejected entirely, others taken readily, *e.g.*, starch grains were never recovered in the alimentary tract, while *Glœocapsa* passed through in great numbers (p. 227). It is permissible, therefore, to postulate that the labial palps behave in harmony with the rest of the food-gathering apparatus.

(*b*) *The Degree of Digestion a Response to Tissue Demands.*

The cilia of the alimentary tract are virtually in constant movement. (Nelson, '18, has reported the partial suspension of the

cilia of the style sac.) It involves little, if any, additional energy to keep a stream of water coursing through it. That much food material passes through without being digested is shown both by the mass color of the feces and by the appearance of diatom and other algal cells from the feces, when examined microscopically. It has been argued that diatoms are accidental and not the real food. Empty diatom tests are found in considerable quantity in fecal matter, and there is no doubt that the contents have been digested out of, at least, some of them. Furthermore, the freshly formed style, in a mussel which has been starved and then fed with diatomaceous matter, has the amber color of diatomin. In this case digestion is prompt and rapid. The style tends to become colorless as soon as the streaming of food through the style sac is checked by the growing style.

Since the ciliary mechanism is functioning constantly, and since some material is digested and some is not, it may be concluded that the demand for food on the part of the tissues affects rather the secretion of digestive fluids and ferments than the control of ingestion.

On several grounds it is seen, then, that a given particle may or may not be digested on its passage through the alimentary tract. Nelson's suggestion ('18), that the style sac serves as a means of returning undigested particles to the stomach where they may be exposed again to the digestive secretions, is very plausible. The morphology of the structure fits such a function to a nicety. Moreover, while a starved mussel is renewing its style much green material is being threaded into its core. The fact that some diatoms are digested while others are not might at first thought find a sufficient explanation in Nelson's view. For some particles would be diverted into the style sac and others continue on undigested. This would perhaps as readily explain the presence of both normal diatoms and empty tests in the rectum as my suggestion of uninterrupted feeding. There is no doubt that Nelson's explanation is entirely adequate for *Ostræa* and *Modiolus,* for these forms interrupt siphoning and feeding with every tide. Their styles are absorbed and renewed regularly with the ebb and flood. Hence twice each day the style sac is

thrown open to the passage of food; and for this reason, if for no other, one should find a mingling of digested with undigested food in the rectum. In the Unionidæ this mingling does not have an adequate explanation in the style sac. For there is no loss of crystalline style except with starvation. No normal mussel is found without it. Under normal conditions the style has no color, with a very slight core of green. The amount of food which passes through the style or style sac under ordinary conditions is very slight. Hence most of the digested diatoms whose tests are found in the rectum have been through the stomach but once.

Résumé.—In the rectum and feces the simultaneous occurrence of green diatoms and empty tests shows that part, but not all, of such material is digested. At any rate it is usable as food. Ingestion is continuous, but digestion is discontinuous, and dependent upon demand for nutrition on the part of the tissues. In the Unionidæ the return of material from the intestine to the stomach through the style sac does not account for much of the food actually digested, for such a transference of food is possible only when the style sac is empty. Since digestion is a chemical reaction the contents of the stomach should be affected alike, and not some wholly digested and some not at all, when equally digestible.

3. The Utilization of Nannoplankton and Megaloplankton.

The materials recovered from the rectum grade down from the largest phytoplankton to very small species. While stomach and rectal contents vary, the larger particles are obviously oftener found undigested than the smaller. This suggests that the smaller are more easily digested; that everything else being equal, digestibility is inversely proportional to size. The question arises: does the nannoplankton constitute an important, though less conspicuous element of the food of a mussel?

Juday (see Ward and Whipple, '18) has shown that the nannoplankton content of a lake may exist in vastly greater number and in a much greater volume than does the net plankton. Even though most of such organisms should escape through the gills

of the mussel, the recovery of only a fraction of them is sufficient in some lakes to equal or surpass the total volume of larger organisms utilized.

(*a*) *Experiments in Selective Feeding.*

A few experiments were made to separate the nannoplankton from the grosser, in order to feed them separately.

There is no hard and fast line by which the nannoplankton may be distinguished, except Lohmann's ('11) arbitrary size limit of 25 microns. Stomach and intestinal contents were examined for evidence of a predominance of either larger or smaller forms. So far as the diatoms are concerned such evidence is not very conclusive, though on the whole the smaller organisms apparently have the better of the argument. As for Flagellata, they were commonly seen in the rectal contents, but more often in the stomach. This is as we should expect, for some of them do not possess so resistant a test as the diatoms.

The fact that even a few of the smaller flagellates and diatoms are found demonstrates that the gill-meshes are fine enough to accomplish something with the nannoplankton, while the diminished number of flagellates in the rectum implies the more complete digestion of that group. Certain flagellates are more resistant to digestion than others, *e.g., Peridinium*; some are doubtless of more frequent occurrence in the alimentary tract on account of their colonial form and greater bulk, *e.g., Pandorina.*

We have no very efficient mechanical means for separating plankton according to size. The nets of finest bolting silk allow nearly all flagellates and all but the large or colonial diatoms to pass through. It was found that water, poured through a net suspended in the air, forces more large organisms through the meshes than in the case when the net is suspended in water, and the water containing plankton poured through slowly.

A concentration of net plankton actually took place in the plankton bucket. A complete separation of coarse from fine plankton is not claimed, nor was it necessary to the experiment. Undoubtedly some of the grosser forms passed through the silk. But these were never in sufficient quantity to be detected in the

intestines of mussels feeding in the escaping water. One complete experiment follows:

Water from the creek or lake was first poured through a copper gauze in order to remove such debris as plant fragments and flakes of limy incrustation. From these it passed into a conical plankton-net of No. 20 bolting-silk, terminating in a detachable Birge bucket. The lower portion of the cone and the bucket were suspended in a jar of water. Measured quantities of the natural water, varying from 50 to 250 liters in the several experiments, were passed through. From time to time the meshes of the bucket became choked and the process was slowed down. This was especially true in the lake following high winds, when there was considerable turbidity. At intervals, therefore, the bucket was removed, rinsed out into a container, and the gross filtrate brought into contact with a group of mussels which had been starved for a few days in order to cause the disappearance of the crystalline style. The overflow water which had passed through the silk was allowed to siphon over into another jar in which were kept another group of starved mussels. They were thus given opportunity to feed upon nannoplankton almost solely.

In order to demonstrate that any regeneration of the crystalline style that might occur could not be ascribed to the chemical or physical character of the water itself, a quantity of the strained water was also siphoned over into a sheet of filter paper and mussels placed in the jar beneath. The mussels fed upon filtered water in no instance showed the least evidence of re-forming the style. That many organisms passed through the plankton net is well shown by the amber-green coating which soon formed on the filter paper. Upon these organisms the second group of mussels had opportunity to feed, provided the gills might be a sufficiently effective mechanism to entrap particles which had passed through the silk.

During the progress of the experiment, usually about four hours, checks were kept in the lake or creek, near the experiment. They also had been starved for the same length of time, in tap water.

The water was taken from a depth of two to three feet in the lake, along the east shore, where it is open to wind and wave

action, but has the shelter of a broad zone of *Potamogeton*. The creek water was from the mouth of Pocahontas creek, having a depth from a few inches up to more than a foot, and a bottom of gravel and rubble. A drain from a large septic tank enters 500 feet upstream. In its upper course it receives the water of drainage ditches in swamp land, and has a small, though constant, volume throughout summer. Its flow is rapid, and the character of the bottom such that mussels have not ascended it. The water has a slightly brown color due to its swampy course (Rice, '16).

In this experiment two mussels were used as checks, three kept in filtered water, ten placed in the overflow from the plankton net, and ten were fed upon the gross concentrate given them in tap water.

Of the two checks, one developed a well formed crystalline style, the other none, but it had a large amount of green material in the intestine, showing that it also had resumed feeding.

Of the ten fed upon net plankton, seven renewed the style more or less completely, three not at all; of the nannoplankton-fed eight had formed a style and two had not. These data are not full, yet they demonstrate the ability of the respective food materials to renew the style.

It will be seen that there is little difference in the power to renew the style between the larger plankton which remained, and the smaller which went through the silk. It is certain that the larger forms do have a food value, for there were few of the smaller which remained in the plankton bucket, and the residue of grosser material alone was put into the aquarium with the first group of mussels. It is possibly not quite so well demonstrated that the other eight renewed the styles in response to the ingestion of nannoplankton solely, but such is a reasonable supposition. Two things at least cannot be gainsaid: (1) there was a significant increase in the ratio of megaloplankton to nannoplankton within the bucket, and of course a reversal of this ratio outside, without greatly affecting the crystalline style renewal in either case; (2) no net plankton was recognized in the intestines of the second group of experimental animals, although conspicuous in those used as a check.

The above facts do not argue a greater digestibility of the

smaller organisms. But when the matter of dilution or concentration is taken into account, we have such an argument. The greatly concentrated net-plankton was fed in a container of water which was changed but slightly throughout the experiment. The nannoplankton, on the other hand, was caused to stream over the second group of mussels in the original water, and with the original concentration. If it had been concentrated as much as the net plankton a much more marked ingestion would probably have been obtained. It is hoped that in the future a method for concentrating nannoplankton in quantity may be applied to the solution of this question.

An experiment similar to the foregoing consisted in placing starved mussels in the creek, enclosed in a tight metal container, whose two ends were then closed with bolting-silk. The creek was dammed on either side of the container, so as to raise the level slightly and maintain a flow of water through it upon the mussels. The stopping of the meshes of the silk was expected to interfere with the flow after a few hours. But, as a matter of fact, there was sufficient eddy and overflow to keep the silk fairly well washed, so that at the end of the experiment a slight current might still be detected.

Experimental mussels and checks were placed both above and below the sewer outlet, where they were allowed to feed for twenty-four hours. Small numbers were used here—four mussels in each situation. So far as the results derived are trustworthy, they show a utilization of nannoplankton as food, and corroborate those of the previous experiment.

The mussels from the three situations showed a well-defined gradation in the reconstruction of the crystalline style: (1) those from experimental conditions showed only a partial renewal; (2) the checks nearby had virtually completed the renewal; (3) below the sewer outlet the checks had well formed and entirely hyaline crystalline styles. Presumably they had an additional source of food—the sewer.

The mussels used as a check, and whose styles had grown large and hyaline, accumulated considerable masses of green in the intestine during the twenty-four hours. Examination showed this material to comprise, among other forms, *Navicula, Oscil-*

latoria (small amount), *Scenedesmus, Synedra,* and *Tabellaria.* Considerable debris, both organic and inorganic, was found, and some fragments of considerable length.

In the case of those fed upon "bolted" water considerable amounts of green were found in the intestine, and the styles were flaccid and green. No large particles were obtained. *Tabellaria, Diatoma*, and *Navicula* occurred, but it was only very small species in each case, and no coherent members of colonies. Very little inorganic stuff was found, but many organic fragments, some of them partially digested. The smaller flagellates were proportionately more numerous than in the checks. Naturally the diatoms were of the solider, creek type, and none of the graceful lake forms adapted for flotation.

(*b*) *The Feeding of Sewage.*

The presence of Oscillatoria may be taken as an index of the amount of sewage in the food. It also shows a well-defined gradation in the several feeding stations: (1) no *Oscillatoria* was recorded from the experimental animals above the sewer outlet; (2) very little appeared in the checks kept in the unscreened stream at this point; (3) somewhat more *Oscillatoria,* consisting of very incomplete filaments, was seen in the experimental animals below the sewer; and (4) the checks below the sewer contained numerous large fragments of *Oscillatoria,* and many relatively large bits of debris never met with elsewhere.

Mussels which had been kept in the mouth of the creek for several weeeks prior to these experiments were opened at the same time. The styles were well formed and without color. On various occasions the styles of the experimental animals reacted differently. At one time all of the styles were whitish when partially renewed, having a distinct white, spiral core. In those most perfectly formed the white color was disappearing, and the entire mass was becoming more solid and more hyaline. On other occasions the newly formed styles were of the typical amber hue which suggested diatomin. Subsequently mussels opened here contained sometimes whitish, sometimes colorless styles. At times freshly formed styles were found which were green in the

stomach and becoming white in the style sac, indicating a change from one color to the other.

Only one explanation of the above differences offers itself—namely the effect of bacteria. These, through a mass effect or through the breaking down of the style substance itself, are probably responsible for the white color. Rice (*l.c.*) has shown that the abundance of bacteria and of nitrates is here subject to profound variation, on account of the periodic discharge from the septic tank mentioned above. Whatever the cause of the white color it might have been expected to be mingled with green from the normal food brought down from above. That green is actually present in concealment is shown by the boiling of such styles (p. 226). The white style pertains mostly to the creek. In the lake a white style is observed only during low water in mussels which have been feeding near the creek outlet. During freshets and great dilution of the bacteria it does not occur even in the creek. These observations may be taken as a further indication of the direct dependence of the crystalline style upon the character of the food.

(*c*) *The Feeding of Infusions.*

Previously I had observed the renewal of the style and the accumulation of material in the alimentary tract of animals fed with hay infusions rich in ciliates (Allen, *l.c.*). The unmistakable finding of protozoan fragments in the stomach showed that some such material is ingested, and I was satisfied that the style renewal was due to their presence. The above nannoplankton studies suggested that there might also be food value in the bacteria and flagellates which are present in such concentration in infusions. The former experiments upon the feeding of infusions were repeated with the same results. Again, white crystalline styles appeared (p. 220). However, in order to determine if the bacteria and flagellates present may be responsible for the style renewal an attempt was made to separate them from the large ciliates. The above method for the separation of plankton was applied here. It was possible, at any rate, to dispose of most of the bacteria by washing. The process resulted in the death of many ciliates due to crushing and to the change into fresher

water. However, it was possible to accumulate a considerable mass of living and fragmented infusoria. Starved mussels fed upon this concentrate did not renew the style. This negative result must not be trusted too implicitly, considering that, as reported above, ciliate material is sometimes found in the stomach. Yet the present experiment points toward a greater utilization of the smaller organisms of infusions than of relatively large ciliates. Aside from the ciliated protozoa the infusorian population consisted mostly of extremely minute forms. The maceration experiments described elsewhere (p. 228) show conclusively that the Unionid gill is capable of intercepting very small material indeed—even the pyrenoids of algæ.

It may be questioned whether the formation of a crystalline style is a reliable index of the feeding activity. In my opinion it is as reliable as a direct examination of the alimentary tract. On some occasions food may be found in the intestine before regeneration of the style is perceptible. But, on the contrary, there are as many occasions when a starved mussel recently fed is seen to have the beginnings of a style before anything is readily recoverable from the intestine. In far the greater number of cases examined the synchronism between style renewal and the presence of food is exact. The bearings of this upon the significance of the style are discussed below (p. 229).

Résumé.—The more minute plankton organisms are of as great nutrient value as the more conspicuous, often undigested matter commonly listed from rectal or fecal examinations. However the net plankton is shown to have a food value as well. Experiments which more or less perfectly separated the net- from nannoplankton show that both are capable of re-forming the crystalline styles of starved mussels. The minute flagellates sometimes exceed the volume of net plankton in lakes many fold. Since it is certain that they can be entrapped by the gills of the mussel and can be ingested, it is likely that their rarity in the rectum is due to the fact that most of them have been digested. Infusions which have nothing of food value except ciliates and minute organisms renew the style.

4. The Physiology of the Crystalline Style.

Everyone, myself included, who has dealt with the crystalline style during the past decade, has made apology for adding to the bulky list of the things not certainly known concerning that organ.

(*a*) *Recent Studies.*

The most thoroughgoing and satisfactory account of the crystalline style is that of Nelson ('18). He has assembled and organized the literature on the subject to the minutest detail, has very effectualy eliminated most of the groundless speculations, and has sifted out the truth contained in the rest. Nelson's work on the morphology is of a sort which virtually closes that subject. All future studies of the crystalline style may well make Nelson's work the point of departure.[2] There is no occasion to review the literature here or to describe either the style itself or the associated portions of the alimentary tract. I shall be content to record the physiological data which have accumulated during the intermittent observations made over a period of some six years.

Most of the writers, with the exception of Mitra ('01), and Nelson (*l.c.*), have taken a viewpoint which has been fatally erroneous, it seems to me. Despite all the divergent speculations which observers have permitted themselves to make (a point well reviewed by Nelson), they have really been looking for *one* explanation—the most plausible function that this organ might be supposed to perform. Few have granted the probability that two or more uses of the style might exist concurrently.

[2] The preceding sentence, when written, was prophetic. For just as this paper is about ready for the press, Edmondson's timely account of the crystalline style in *Mya arenaria* has appeared ('20). Like Nelson, he has devoted considerable study to the morphology, but has centered his attention upon the renewal of the style. It is gratifying to find others interested in the physiological study of the style, for, aside from its chemistry, most work has been done from the viewpoint of structure.

This author's account of *Mya arenaria* shows the style to have diverged very far, indeed, from its homologue in the Unionidæ. It lies in a distinct cæcum. Operative methods instead of starvation were necessary to remove it. It is a very solid structure, nearly insoluble, and nearly devoid of albuminoids. Its regeneration in *Mya* precedes rather than follows resumption of feeding. Seventy-four days were required for its reformation in *Mya*, while one day more or less suffices in the Unionidæ.

Mitra (*l.c.*) was the first to recognize clearly that the crystalline style may meet several needs. The fact that it is dissolved when food is wanting, and that in solution it may be taken up by the blood, leads to the conclusion that it is (so far as it goes) a reserve of nutriment. Furthermore, the work of Mitra and others has shown that it bears enzymes capable of furthering starch digestion. Dr. Scott Edwards has kindly checked over this matter for me, with confirmatory results. Insofar as suitable food is brought into contact with it, it is a means of supplying digestive ferments.

Nelson (*l.c.*) has shown very well that the rotation of the crystalline style against the "gastric shield" in the stomach shreds the dissolving end so as to form a brush. The rotation of this brush sweeps the food into the proper ciliated channels, aids in dissolving the food out of the mucous masses in which it reaches the stomach, and acts as a substitute for peristalsis in mingling food with the digestive fluids from the liver, etc. It might have been pointed out that these movements afford a ready means of bringing the contained enzymes of the style into thorough contact with the food.

Since the style actually accomplishes all these things, we can not choose any one of them as the function for which it was designed. We cannot assert that the style is exactly adapted to perform any one of them, or that it is the function which it has always performed in ancestral forms. If such were the case one might expect to find somewhere in the more primitive existing species a style little changed from the ancestral condition. But the Najades have taken to fresh water, and as a result have become profoundly modified in life history, ontogeny, and structure. While the crystalline style has shown as little structural change as any organ, it is not improbable that its relation to the organism as a whole, to metabolism, has suffered changes of which we know nothing.

Nelson suggests a further function of the style sac, that of returning undigested material from the intestine to the stomach, to prevent the loss of food. In *Modiolus,* which undergoes a periodic cessation of feeding and dissolution of the style, the

cilia of the sac periodically carry a stream of food from the intestine through it into the stomach. In common with all ciliated epithelia this organ raises the very interesting and equally difficult problem of accounting for the present direction of the beat of the cilia. In the simpler ancestral forms the beat most probably was in the opposite direction.

The style sac of *Modiolus* contains more food in winter than in summer. It is difficult to see why the structure in *Modiolus* should be (as Nelson reports) more effective in winter, when the metabolism is low and the food requirement slight, than in summer, when the demand for food is greater. In winter the secretion of the style substance is slowed down by the temperature to such an extent that the organ is not promptly re-formed with each feeding period (p. 229). The style sac therefore contains no style and may be utilized to reconvey food from the intestine to the stomach. In the Unionidæ it is certain that the return of particles through the style sac is a phenomenon which takes place normally only after starvation. Of the many hundreds of specimens examined when taken from the water, not half a dozen were ever found in which the style was lacking.

Usually the newly formed style has an abnormally large core of plankton. This indicates that the first undigested or partially digested material which streams into the intestine is diverted at the posterior end of the style sac and carried forward again into the stomach. In the meantime the glands of the typhlosole (Nelson, *l.c.*) begin secreting and wrapping the spiral of the style substance about this core. The streaming in of materials from the intestine is limited more and more as the style more and more completely fills the lumen of the sac. After the style moves forward and has been dissolved its entire length, the newer portion, with a diminished core, has entirely replaced the original portion with its loose structure and large core of food. This takes place twice daily in *Modiolus* or *Ostræa*, and the newly regenerated style is thus oftener encountered. In *Lampsilis* or *Anodonta* under normal circumstances, it is a very infrequent occurrence. My observation has been to a very great extent upon lake forms, which are not subject to many vicissitudes. It is

presumable that river forms, if taken at the right times, demonstrate a more periodic activity in response to the rise and fall of the current, the degree of turbidity, etc.

Nelson is probably in accord with these reservations concerning the Unionidæ, for he agrees (*l.c.,* p. 100) that the formation of the style is directly dependent upon the food supply. In the marine forms the return of food through the style sac to the stomach is considerable. In the Unionids the return of food through the style sac has become reduced because the sac is usually occupied by the style.

The spiral character of the style, caused, as demonstrated by Nelson, by its axial rotation, has been brought out nicely in three ways in my observation:

(*a*) A regenerated style in one starved *Anodonta* was found to have a great deal of green matter throughout, and but a very slight amount of style substance. It had grown to at least normal diameter. When kept for a time the secreted portion dissolved out, leaving the green cohering portion wrapped about the core like the threads of a screw, or a lathe shaving.

(*b*) A few whitish styles were boiled for a short time in strong sodium hydroxide. They were much reduced, and the residue was in the form of a close rope-like spiral.[3] The white appearance had given place to the amber-green color characteristic of most newly regenerated styles.

(*c*) The actual rotation of the style has often been observed.

Examinations were frequently made of the food substances which could be recovered from the core of more or less completely regenerated styles. The food never showed a perceptibly greater degree of digestion at the stomach end than posteriorly. It is not likely, therefore, that the digestive processes are much furthered during progress through the style or style sac toward the stomach.

(*b*) *The Feeding of Specific Substances.*

A series of experiments in feeding certain substances, and in forced feeding by injection into the stomach, were undertaken

[3] Edmondson's ('20) figures of partially formed styles very well represent these.

to throw light upon the actual stimulus which initiates the renewal of the style. The stimuli may be either mechanical or chemical.

Carborundum, carmine, starch, etc., of varying fineness, were introduced into the incurrent siphon with the streaming water. Such organic or inorganic material, however neutral, of whatsoever dilution, or however administered through the respiratory water, were never found subsequently in the alimentary canal. Nor did the molluscs ever display any indication of style renewal in response to these things. The experiment has a further significance to be discussed on page 229.

A culture of *Glœocapsa* was looked over carefully and found to have very few organisms of the size of *Glœocapsa,* but much coarse debris. This material was washed into a jar with active, starved mussels, and agitated from time to time to prevent its settling. After eighteen hours the mussels were examined. The crystalline styles were partially renewed in all cases. In others kept in jars of *Glœocapsa* not stirred frequently the styles failed to show any indications of re-forming, even after three or four days. Evidently not enough food to stimulate style formation had been taken into the siphons. In all, small masses of *Glœocapsa* were encountered in the rectum, in the stomach, and in clots of mucus upon the gills and palps. The clots were almost pure *Glœocapsa.* The stomach and intestine contained minute fragments of green, partially digested individuals, and sometimes *Glœocapsa* cells without the capsule. There is thus no doubt but that a pretty rigid selection of the alga from the coarser matter with it was taking place, and that the alga was being digested.

The frequent occurrence of the *Glœocapsa* in the rectal contents, still wrapped in mucus, shows the effect of the want of the style. Had that organ been present the mucus masses would probably have been torn up, the alga freed in the stomach and exposed to digestive action. It appears that the process of digestion does not function perfectly, even prior to the formation of a style, and not even a hungry mussel exposes all particles equally well to the digestive fluids.[4]

[4] Edmondson finds the alimentary tract of *Mya arenaria* empty of food until the style is partially replaced.

A quantity of *Spirogyra* and other filamentous algæ was cut as finely as possible with scissors, then macerated with a pestle. Examined microscopically there were found fragments of cell-wall varying in size, fragmented chloroplasts, and pyrenoids. Without screening, this material was fed, in considerable quantity to starved mussels. The water was agitated occasionally so as to keep some of the macerated material in suspension. One mass of alga had been taken from a dish in which decomposition had gone far. Although the mussels held the siphons nearly closed in the decomposing culture, they nevertheless ingested sufficient alga to answer the purpose of the experiment. Feeding the decomposed alga is comparable to the feeding of infusions, and the animals reacted similarly. In all cases of feeding infusion, decaying alga, and the feeding of mussels below the sewer outlet in Pocahontas creek, the regenerated crystalline styles had the same milk-white appearance. Thus there is no doubt that in all cases the color was due to bacteria. Mussels fed upon macerated fresh *Zygnema* had clear, and colorless or green, styles.

In one mussel fed upon a maceration of *Spirogyra* the partially regenerated style had a large core, and only two or three thin layers of style substance. This gave it the proportions and appearance of a rubber tube. When stretched out in a watch crystal and placed under the weight of a cover glass, the contents slowly oozed out at a broken point. The core was then seen to consist almost entirely of pyrenoids (or like bodies) closely packed, and in very great quantity. Their mass had a gray-yellow color. Only here and there was there a minute spot of green. Not a trace of cell walls or of a spiral fragment of chloroplast was found here or in the stomach. We have then another case of the rigid selection of food particles, and a little suggestion of the character of the materials which are capable of inciting the secretion of a new style. Again we have evidence that the gill is capable of taking very small particles from the water.

In a decaying macerated *Spirogyra* culture several starved mussels kept the siphons almost entirely closed. When a few c.c. of alcohol were added they shortly began and continued siphoning vigorously. Yet the food sorting mechanism functioned normal-

ly, for no stomachs nor intestines were found to contain larger fragments than usually occur there.

(*c*) *Forced Feeding.*

From the above experiments, and on grounds discussed elsewhere (p. 227), it is seen that the ingesting apparatus exercises considerable choice, and that (at least under experimental conditions) only certain sorts of material are admitted to the stomach.

An attempt was made to introduce distasteful matter into a starved mussel with the food. When fed alone, carmine had never been ingested. It was therefore administered with *Glœocapsa* and macerated *Spirogyra*. In no case was it found in the alimentary canal. Very little of the food entered, for that matter. The presence of the carmine caused a rejection of most of the food as well.

In order to ascertain if substances rejected by the mouth might yet have the power to stimulate the secretion of the crystalline style, these were introduced little by little through a fine pipette directly into the stomach. Fine carborundum (120–180 gauge) carmine, and starch were tried. In none of these cases was any trace of a style to be found later. It should be explained that the shock of operation was not alone responsible for this failure, for when *Glœocapsa* was fed to the animals in the same way, it was capable of renewing the style to a slight degree. There is sufficient ground for the conclusion that mere mechanical stimulation of the intestine or style sac on the part of fine particles is not sufficient to initiate the formation of the style. There must be a stimulus of a chemical nature as well. A reaction to the feeding activity might have incited secretion through reflexes from the palps. Yet this is inadequate to account for the renewal of the style when *Glœocapsa* was administered through the stomach wall.

(*d*) *The Effect of Temperature on Style Renewal.*

With the approach of winter it becomes more and more difficult to secure a prompt renewal of the crystalline style on the resumption of feeding. Where experiments are made in water of quite low temperature the same behavior is observed. Riddle,

('09), Krogh ('14) and others have shown that the rate of metabolic processes is related to body temperature. In this case the decrease in temperature probably directly affects the rate of secretion of the typhlosole glands. It is not improbable that a seasonal metabolic rhythm exists.[5]

In order to test the effect of temperature alone in this matter and to eliminate the other possible elements, the following experiment was carried out:

Checks were kept at room temperature. In each repetition of the experiment one jar was placed in the cold water of the outlet of an artesian well. When the temperature was sufficiently reduced the experimental starved animals were introduced into their respective jars. Meantime concentrations of lake plankton were made by filtering through fine bolting-silk, and the plankton remaining in the bucket washed out into the check and experimental jars. Each mussel had a jar to itself. The water in all had a decided green tint when agitated, for the concentration was in all cases from 100 volumes to 1. The jars were well shaded to eliminate the possible action of sunlight in orienting the planktonts, and thus keeping the food equally available to all. The amount of water was equalized, and the mussels so placed that the exhalent stream played obliquely upon the sides of the jar and maintained an eddy to keep the planktonts in circulation.

The material was collected at about 10:00 A.M., and about three hours were allowed for the temperature adjustment. The experiments began at about 1:00 P.M., and continued from three to four and one-half hours, usually four. The average temperature of the experimental jars at the beginning of the experiments was 13.4° C., varying between 12.6° C. and 14.0° C. The average of the same jars at the end of the experiments was 13.1° C., varying between 12.0° C. and 14.4° C. There was an average loss in temperature of 0.3° in these jars. The checks gave an average at the beginning of 26.0° C., varying between 23.6° C. and 30.0°; at the end an average of 27.2° C., and a variation between 24.0° and 29.6° C. The average rise in temperature of

[5] The crystalline style of *Mya arenaria* is reformed more rapidly during summer than winter. Edmondson (*l. c.*) ascribes this to the increased metabolism of the approaching breeding season.

the checks was 1.2° C. At the beginning the checks averaged 12.6° higher than the others, and at the end averaged 14.1° higher. Nearly ten degrees (9.6) separated the lowest check from the highest temperature in an experimental jar. As was to be expected the atmospheric temperatures created considerable variation in the checks (6.4°) and much less in the cooled jars (2.4° C.)

Of the twenty animals used only ten showed partially renewed styles. Of these ten only two occurred at the reduced temperature, and eight in the checks. The two which appeared in the low temperature were smaller than any of the eight formed in the checks. Moreover, the two were both in *Anodonta,* and *Anodonta* has shown a greater response always than *Lampsilis,* never failing to show some renewal. *Lampsilis* more slowly loses and more slowly regains its style than *Anodonta.*

While the number used is small, exact quantitative results are here unnecessary, and there is sufficient demonstration of the qualitative effect of temperature upon style formation. This effect may be partially explainable through the effect of temperature upon the cilia and the rate of ingestion. But the reason mentioned above is probably more pertinent, for the intestine was usually found to contain food in the experimental animals.

So long as the quality of the ingested material is right, the quantity required to initiate the formation of the style is very small. At times a single battery jar of water dipped at random from the surface of the littoral has contained sufficient food to restore it, in part. Held to the light the water had given no hint of green. But after it had been siphoned from one to two hours, the resulting thread-like crystalline style contained a conspicuous core of green.

Where food is abundant the length of time needed to renew secretion after the beginning of feeding is very short. A fair beginning may sometimes be observed within fifteen to thirty minutes. Large well-formed styles are sometimes secreted in four hours or less. The time depends largely upon the degree of starvation. More often twenty-four hours, at least, are required. On the whole it is a much more deliberate process than in some

tidal forms, where the breaking down and renewal of the style occur rhythmically.

Of passing interest is the observation that small, newly formed styles sometimes may be seen coiled up in the stomach, where they have pushed forth more rapidly than they could be broken up and dissolved against the gastric shield.

Résumé.—It is here contended that the crystalline style accomplishes a number of purposes, for none of which it is entirely indispensable, nor entirely a perfect adaptation; that it is no longer performing an identical, single, primitive function traceable to a primitive Lamellibranch ancestor. The response of this organ to similar conditions is much the same in various bivalves; but the tranquil life of the lake has stabilized the feeding activity and the style formation in the Unionids, while the styles of some species inhabiting the marine littoral are profoundly affected by the tidal phenomena.

The formation and dissolution of the crystalline style goes on in the same way that a paper, candle-lighter might, if extended to an indefinite length by rolling up a sheet of paper of indefinite length, and burning off the free end as rapidly as new paper is added at the other.

There is no evidence that digestion is furthered during the passage of food through the style or style sac.

The feeding of inert substances, both normally and through the stomach wall, indicates that the mechanical stimulation of the wall of the enteron is not alone the cause of the secretion of a new style. The rate of formation of the style is shown to depend in part upon temperature. Little food and little time are required to set the process going.

5. The Mechanism of Ingestion.

It has long been known that the gills with their great multiplication of surface are responsible for the movement of respiratory water, and for the concentration of food material from the water. It is surprising to encounter in the work of so eminent a student as Simpson, written only a score of years ago ('99), a statement that the siphoning is due to the waving of the palps.

It was shown by Posner ('75), Wallengren ('05), and others, against the contention of M'Alpine ('88), that the collections of food are transmitted to the labial palps, and by their cilia to the mouth. The writer shows (*l.c.*) that the ciliary streams of the upper portion of the mantle chamber all tend toward the mouth; while those of the lower portion of the visceral mass and mantle lead away from the mouth. The latter accomplish the duty ascribed by M'Alpine to *all* the cilia, that of freeing the mantle chamber of heavier materials and rejected food clots.

It was stated by the writer that the food material is subject to rejection at four points: (1) the siphons, (2) the point on gills and mantle where the food stream passes to the palps, (3) the furrowed surfaces of the palps, and (4) the lips, at the mouth. More recent observations have all corroborated this. Perhaps the fact has not been sufficiently stressed that only an unusual chemical or tactual stimulus results in the closure of the siphons or lips. The palps somewhat oftener refuse masses from the gills and mantle by turning aside. The greater number of reactions occur as the food stream passes between the contiguous palp surfaces.

The work of Wallengren ('05), and others, has demonstrated the action of the labial palps, the ridges of which are capable of reversing the food stream. Near the distal margin of each ridge of the palp surface the beat of the cilia is toward the apex, both in front and behind. When the ridges are inclined forward, the effective beat of the cilia is forward; when the ridges alter their axis, the backward-beating cilia are brought into play and the others turned under.

The course of the ciliary streams at the bottoms of the furrows between the ridges is much more difficult to observe. Wallengren believes that the cilia strike downwards to the edge of the palps, and that the resulting streams belong to the excurrent system. Siebert ('13), working on *Anodonta cellensis,* says they strike in the opposite direction—upward to the apex of the inverted V formed by the two palps, thence forward to the lips and mouth.

(a) *The Rôle of the Labial Palps.*

The writer has checked the matter as carefully as possible, and believes that there is ground for the views of both Wallengren and Siebert. The details of arrangement may not correspond exactly in the several species. On the lower half of the palps the cilia under consideration usually strike downward, and those of the upper half strike upward. Thus the lighter and finer particles tend to be drawn upward and forward as food, while the heavier, coarser, materials are more likely to be carried downward. The differentiation of the mechanism corresponds pretty well to that of the upper and lower portions of the ciliated surfaces of the mantle chamber as a whole. It is impossible to make direct observation of the streaming on any one ridge of the palps. But where substances of varying fineness are placed together on the palps, such as carborundum dust and carmine, there is a tendency to assort them. The carborundum particles move along the apices of the ridges and are carried nearly lengthwise of the palps. The carmine gravitates farther into the furrows between the ridges. Near the lower margin of the palps carmine is carried obliquely downward and forward, and on the average reaches the lower edge of the palps before the carborundum. When placed on the upper portion of the contiguous palp surface, carmine is drawn upward and forward to a greater extent than the carborundum, then forward toward the mouth.

The respective upward or downward pull upon the carmine may be accentuated by stretching the palps lengthwise, thus drawing the ridges farther apart and exposing the cilia of the furrows to a greater extent.

Attempts were made to effect a reversal of the ciliary currents of the furrowed surface of the palps by injections of curari, strychnin, atropin, pilocarpine, and by electric stimulation. The injections were made through the body wall into the sinuses near the base of the palps. Observations were made at various intervals from ten minutes up to several hours after injection. It was never found possible to control the reaction. There was a perceptible response to none of the several drugs except strychnin. This sometimes caused a contraction, at other times a relaxation

of the palps. When the palps were in a state of contraction, most of the streaming carmine and carborundum were drawn to the edge and into the mantle chamber. These meager results tend to corroborate previous observations on the reaction of the palps—that food may be rejected through a greater or less erection of the ridges. Negative results prove nothing, while ever so slight positive evidence may be taken as an indication that a reversal of the ciliary streams can, and actually does, take place, through the bringing of another set of cilia into play.

The above shows clearly that the palps bear two sets of cilia working at right angles to each other. Due to their interaction materials often travel obliquely downward or obliquely upward. The former materials are eliminated at the lower edge of the palps, the latter reach the mouth.

The effectiveness of the assorting mechanism is well brought out by the measurements of the ingested particles. The largest fragment I have ever secured from the enteron was found in the intestine—a pinnately branched alga 3.3 mm. in length, probably *Myxonema*. The second largest particle was a bit of *Oscillatoria*, 1.5 mm. in length. It is unusual in Winona Lake mussels to encounter fragments of greater length than 500 microns. Starved individuals, probably experiencing a sensation akin to hunger, are observed to ingest freely much more large material than under normal circumstances.

Nelson ('18) has described the action of the food sorting cæcum of *Modiolus*, a diverticulum of the stomach. Since *Modiolus* ingests large quantities of sand in its periodic feeding, *Nelson's cæcum* affords a means of separating food from sand. It has been shown elsewhere in this paper that feeding in the Unionidæ is a more constant function, and that little sand and mud are taken into the stomach. The gills and labial palps are an entirely sufficient assorting mechanism.

(*b*) *The Gills as an Assorting Mechanism.*

Little attention has been given to the gills as having a possible food-sorting function. I find that they play no small part. Clots of mucus taken from various parts of the gills and palps have

been examined, and often contain little but the finest ingestible material. This was well shown in the case of a *Glœocapsa* culture fed to a starved mussel. The culture was very pure except for numerous fragments much coarser than the *Glœocapsa* itself. There was an almost complete separation of *Glœocapsa* from the other material by the gills themselves. Almost all the larger fragments had been separated out by the gills themselves before the finer had been agglutinated in mucus. Little of the former was found in the masses present in the alimentary tract.

The marsupial function of the gravid gill of the female interferes somewhat with its respiratory and food collecting functions. Ortmann ('12) has shown that secondary water tubes appear, in which water circulates about the egg masses, and accomplishes the aeration of the eggs and glochidia. Yet the volume of water siphoned is much less than in the case of the non-marsupial gills. This is well brought out by the fact that the gravid females almost invariably regenerate the crystalline style much more slowly than others. When kept under artificial conditions for some time the gill-masses are usually aborted, another indication that the gravid gills are unable to meet all the demand upon them. The greater remoteness of the marsupial gill has suggested that it has become differentiated for the storage of the eggs and has lost its food collecting function. This notion is pretty well refuted by the facts mentioned above concerning the regeneration of the style in gravid females as compared with non-gravid females. It has also been suggested that the mantle has taken over much of the respiratory duty of the gills. If this were true the gravid female should be under no special respiratory difficulty. When first brought into captivity these females die at a much greater rate than others. The accessory water-tubes seem to be a very imperfect makeshift, sufficient perhaps for the glochidia, but affording the mother little aid.

Since in the gills and palps there exists a mechanism well adapted for the sorting of food; since both observationally and experimentally this mechanism is shown to accomplish a concentration of food; and since the contents of the alimentary canal have a decided green or brown color due to such concentra-

tion, we may feel safe in the reiterated conclusion that the Unionids exercise choice in the ingestion of materials. As stated by Zacharias ('07), Petersen ('11), the writer ('14), Baker ('16), and others, considerable quantities of inorganic and organic debris are carried into the stomach with food. Probably much of the stuff which Evermann and Clark ('17) call "mud" is organic. The fact that neither they nor other writers list *sand* in the stomach contents is further evidence of a selection of food material, and that river species are not an exception. Starved mussels were placed in the lake in two localities—(1) an open leeward shore in clear water; (2) near the outlet of Pocahontas creek, in muddy water following a rainstorm. The mussels of the first situation reformed the crystalline styles within a few hours. The others contained great quantities of muddy mucus, and did not have well renewed styles until the following day. The slow renewal of the style may be accounted for in part by the dilution of the food. But the presence of mud must be held partially accountable, for immediately after the subsidence and clearing of the water it was always found to contain ample food material to renew the style promptly.

In most species the position of the siphons at some distance above the substratum tends to keep out most of the grosser particles, admitting little but plankton and other materials in suspension.

In the discussion of the crystalline style (p. 227) the feeding of specific inert substances were recounted. When such materials which were readily identifiable were admitted with the incurrent water they were in no case found in the alimentary tract. As far as size is concerned these particles could very readily have entered the mouth. Since all were rigidly excluded we cannot doubt that sense organs exist for their detection, and that the assorting mechanism is a fairly effective one. Of the materials mentioned only starch might be expected to have a food value, though we cannot assume that it is in acceptable form. As a matter of fact the rejecting reactions were more vigorous in response to starch than to the other substances.

In the above experiments on the crystalline style neutral sub-

stances were introduced through the body wall into the stomach. At later periods the intestine and rectum were opened. Carmine and starch grains were recognized throughout the length of the alimentary canal. Only a few carborundum flakes were found in the intestine, and the rest were not carried out of the stomach. It is thus shown that the ciliary streams of the intestine are capable of manipulating only minute particles. The cilia are too small or too sparse to take care of the 120-gauge carborundum, even in suspension in the liquid of the gut. Thus they must be altogether inadequate to keep a stream of sand in motion, if sand were ingested, unless it were of extremely fine grade.

(c) *The Marly Incrustation of the Shell.*

Since the dense, limy incrustation deposited on the exposed portions of the shells of lake mussels is the site of the active proliferation of diatoms, I suggested (*l.c.*) that this might be a source of food. In order to test its food value the following simple experiment was made:

A number of freshly collected mussels were placed in an aquarium; an equal number, having the incrustation scraped and washed off, acted as controls. During intervals, covering several days the animals were opened and the condition of the crystalline styles noted. The experimental animals were found to contain a trace of the style up to the fifth day, while the checks had virtually lost it by the end of the second day and entirely lost it on the third. Of course the crowding created rather special conditions, unlike those of the lake. The conclusion is that the incrustation contains considerable food.

6. Summary and Conclusions.

1. Feeding in the Najades is a nearly constant function under normal conditions. The presence of much undigested and sometimes living matter in the rectum and feces shows that there is a greater fluctuation in the degree of digestion than in the rate of ingestion.

2. The posture of a mussel has no effect upon the continuity of the feeding process, a further indication that under normal

circumstances ingestion may go on with less effort than an interruption of feeding.

3. The return of undigested material from the intestine through the style sac to the stomach is an unusual occurrence in the Najades, which takes place only after periods of starvation, and which is interrupted with the reformation of the style. It is a function much less significant in the Najades than in the tidal forms.

4. Experiments in feeding relatively finer and coarser plankton show that both are capable at least of stimulating the renewal of the crystalline style. Both have food value. It is probable that the nannoplankton furnishes a much greater part of a mussel's food than has been suspected. The studies of intestinal contents of the Unionids have not demonstrated what the *actual* food is, but rather the undigested residue. Experiment here has shown, however, that the organisms undigested in the feces are sometimes digested, under another set of conditions.

5. Starved animals fed in Pocahontas creek below the outlet of a sewer showed the following peculiarities in intestinal contents: (1) the occurrence of many relatively large organic fragments; (2) abundance of minute flagellates; and (3) great quantities of *Oscillatoria* filament.

6. The regeneration of the crystalline style is in response to the ingestion of food, and not due to the physico-chemical character of the water.

7. Creek-fed mussels show a variation in the color of the style through hyaline, amber, and milky. The apparently rhythmic character of this variation corresponds roughly to the variation of sewage discharged from a septic tank. The milky color is accounted for by the presence of bacteria.

8. A repetition of experiments in feeding infusions indicates that flagellates (and bacteria) present are responsible to a greater extent for the renewal of the style than are the bulkier ciliates.

9. The reformation of the crystalline style is a satisfactory index of the renewal of the feeding activity.

10. The function of the crystalline style varies. It may more or less imperfectly perform several functions at once. The

rhythmic loss and renewal of the style in tidal forms has no parallel in freshwater species.

11. The feeding of specific substances in high concentration never produces a renewal of the crystalline style unless such subtances have a food value. No indication was observed that the stimulus for its secretion is a mechanical one.

12. The style is much less readily formed in autumn or winter. That temperature is responsible is shown by experiments in which starved mussels were fed a concentration of plankton in water of high and low temperature, respectively.

13. A very small amount of plankton is sufficient to stimulate style formation. Also only a short time is required.

14. The labial palps are the primary assorting mechanism. The gills are of considerable importance in this matter also.

15. Sand is never ingested, at least by lake forms, and mud but slightly. Much less inorganic debris finds its way into the stomach than would be the case if selection were not exercised by the gills and palps.

16. The cilia of the alimentary canal are unable to move coarse materials, or to maintain a stream of sand or heavy mud.

17. Gravidity of the gills is a serious hindrance to the respiratory and alimentary activities.

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BIOLOGICAL BULLETIN

INTER-PERIODIC CORRELATION IN THE ANALYSIS OF GROWTH.

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I. INTRODUCTORY.

In the literature of growth, mathematical equations to describe changes in the actual size of the organism, or changes in the growth rate, are finding continuously widening applications. One has merely to refer to the papers by Robertson, Miyake, Moeser, Ostwald, Reed and Holland (1919), and Reed (1920)[1] for illustrations.

The criticism usually directed against such work is that in the higher organism, growth is a highly complex process, and that in consequence it cannot be represented mathematically. It is because of the very fact that growth is a complex process that mathematical analysis of the experimental data is necessary. Corollary to this must be the recognition of the fact that since growth is not a simple process, no one mathematical formula will be adequate for full description[2] and no one method adequate for complete analysis.

Our purpose in the present note is to illustrate on a series of data collected by one of us (1919) the application of inter-periodic correlation coefficients to certain phases of the problem of growth.

Before passing to the analysis, which is the special purpose of this paper, definition of the terms which will be used and a note

[1] Citations of literature may be traced from Reed's paper.

[2] Those who consider the possible adequacy of a single equation take the ground that if it be possible to represent the growth of an organism by a simple equation, it may be by virtue of the fact that during growth the various (often conflicting external) factors which affect the living substance are integrated by the organism.

on the nature of the data on which the statistical methods are illustrated are in order.

By growth stage we mean any given moment of time at which a series of organisms are measured. It is, therefore, synonymous with age during the growth period. The absolute size of the organism or of one or more of its parts at a given growth stage is the only character of the organism available for consideration.

By growth period we understand the period of time elapsing between the sth and the $s+n$th growth stage.

The increase in size during any such period we shall designate as a growth increment.

By relative growth increment, i_{rs}, we understand the ratio of the growth increment, i, to the absolute size of the individual at stage, r, where r and s are any two successive stages.

Turning now to the question of the original data as given in Table I. of Reed's (1919) publication we note from a study of the physical constants for absolute size in Tables I. and II. that there is an increase in the mean height of the plants up to the 77th day.

TABLE I.

STATISTICAL CONSTANTS FOR SIZE AT VARIOUS GROWTH STAGES.

Growth Stage.	Mean.	Standard Deviation.	Coefficient of Variation.
7	17.931	1.617	9.0
14	36.328	4.786	13.2
21	67.845	8.932	13.2
28	97.672	14.673	15.0
35	130.724	19.174	14.7
42	168.707	24.801	14.7
49	205.397	32.760	16.0
56	229.672	37.842	16.5
63	247.345	42.574	17.2
70	251.776	43.433	17.3
77	253.810	43.767	17.2

The increase from the 63d to the 70th and from the 70th to the 77th day is relatively slight, being only 4.43 cm. or 1.79 per cent. of the height for the 63d day in the first case and only 2.03 cm. or 0.81 per cent. of the value for the 70th day in the second case. The difference between the 84th day and the 77th day is negligible. In view of the fact that there is no appreciable growth in

the sense in which the term is used here between the 77th and the 84th day, this period will be left entirely out of account in the calculation of the correlations for the following discussions.

Furthermore by considering the constants for growth increments as shown in Table II., we note that the coefficients of variation for growth increments from the 63d to the 77th day are abnormally great. This may be in part due to biological causes, but it is doubtless due to a considerable extent to the relatively large error of measurement when the increment is very small in comparison with the size of the organism. If this be true, we should expect the correlations for actual size for the 63d to the 84th day to be about the same as those for the immediately preceding growth stages, but the correlations for growth increments may be expected to be of little value.

TABLE II.

STATISTICAL CONSTANTS FOR GROWTH INCREMENTS FOR VARIOUS GROWTH PERIODS.

Growth Period.	Mean Increment.	Standard Deviation.	Coefficient of Variation.
7 to 14	18.397	3.764	20.5
14 to 21	31.517	5.164	16.4
21 to 28	29.827	7.907	26.5
28 to 35	33.052	7.505	22.7
35 to 42	37.983	11.578	30.5
42 to 49	36.690	14.266	38.9
49 to 56	24.276	16.540	68.1
56 to 63	17.672	13.803	78.1
63 to 70	4.431	4.713	106.4
70 to 77	2.034	5.096	250.5

The problems which may be considered will be presented and discussed seriatim.

II. ANALYSIS OF DATA.

PROBLEM I. *The correlation between the absolute size of the organisms at its several periods of development.*

When examined at an early stage of development, organisms are found to differ among themselves in size. The same is found to be the case when the same series is measured at a later growth stage or at maturity.

In the biological analysis of the phenomenon of growth a prob-

lem of great importance is that of the causes which bring about the differences in size observable at any stage of development, or after growth has entirely ceased. Are individuals which are found to be small at maturity those which were small initially and have remained so from the beginning, or may the growth rate of an individual change during the course of its development to such an extent that it may vary its position in the series under investigation from time to time? That the latter is to some extent the case we know from general observations on human children. The problem to be solved is that of the quantitative magnitude of the relationship between the size of the individual at different stages of development.

The nature of the biological problems to be investigated has been stated in earlier work, and an attempt has been made to solve them by grouping plants according to quintile (Pearl and Surface, 1915) or quartile (Reed, 1919) position in the culture to which they belong and ascertaining the quartile or quintile in which they fall at different stages of growth.

This method has the disadvantage that all the individuals, whatever their size, are lumped together in four or five groups. In this method of treatment, small differences between two individuals are, therefore, given as much significance as large ones, providing they are large enough to throw the two individuals into different quartiles or quintiles.

An alternative method, which will completely obviate this difficulty, is to determine the correlation between the sizes of the individual at different periods of growth. The possible correlations between the absolute size of the individuals in the 11 different stages of growth of the *Helianthus* plants are shown in Table III.

The coefficients in this table can be best understood by first examining those for the relationships between the sizes of the plants near the period of maturity, and then passing to the relationships between the sizes of the plants at earlier stages.

Considering first of all the coefficients in the lower right-hand corner of the table, we note that all the coefficients are very high, denoting practically perfect correlation. This is the relationship which would be expected for a period when the organism has

Table III.

Correlation between the Actual Height of the Plants at the Various Growth Stages.

Stage.	Stage. 7	14	21	28	35	42	49	56	63	70	77
7		+.733 ± .041 17.9	+.558 ± .061 9.14	+.468 ± .069 6.78	+.347 ± .078 4.46	+.193 ± .085 2.26	+.130 ± .087 1.50	+.093 ± .088 1.05	+.069 ± .088 0.78	+.065 ± .088 0.74	+.053 ± .088 0.60
14	+.733 ± .041 17.9		+.889 ± .019 48.0	+.695 ± .046 15.2	+.532 ± .064 8.38	+.343 ± .078 4.39	+.220 ± .084 2.60	+.151 ± .087 1.74	+.150 ± .087 1.73	+.140 ± .087 1.62	+.123 ± .087 1.41
21	+.558 ± .061 9.14	+.889 ± .019 48.0		+.887 ± .019 47.1	+.739 ± .040 18.4	+.552 ± .062 8.97	+.390 ± .075 5.20	+.320 ± .080 4.02	+.311 ± .080 3.90	+.297 ± .081 3.67	+.282 ± .082 3.46
28	+.468 ± .069 6.78	+.695 ± .046 15.2	+.887 ± .019 47.1		+.936 ± .011 85.2	+.752 ± .038 19.6	+.534 ± .063 8.44	+.409 ± .074 5.55	+.356 ± .077 4.61	+.329 ± .079 4.17	+.318 ± .080 3.99
35	+.347 ± .078 4.46	+.532 ± .064 8.38	+.739 ± .040 18.4	+.936 ± .011 85.2		+.892 ± .018 49.5	+.674 ± .048 13.9	+.488 ± .067 7.24	+.394 ± .075 5.26	+.350 ± .078 4.50	+.333 ± .079 4.23
42	+.193 ± .085 2.26	+.343 ± .078 4.39	+.552 ± .062 8.97	+.752 ± .038 19.6	+.892 ± .018 49.5		+.914 ± .015 62.5	+.732 ± .041 17.8	+.629 ± .053 11.8	+.580 ± .059 9.86	+.558 ± .061 9.16
49	+.130 ± .087 1.50	+.220 ± .084 2.60	+.390 ± .075 5.20	+.534 ± .063 8.44	+.674 ± .048 13.9	+.914 ± .015 62.5		+.900 ± .017 53.5	+.819 ± .029 28.1	+.775 ± .035 21.9	+.758 ± .038 20.1
56	+.093 ± .088 1.05	+.151 ± .087 1.74	+.320 ± .080 4.02	+.409 ± .074 5.55	+.488 ± .067 7.24	+.732 ± .041 17.8	+.900 ± .017 53.5		+.948 ± .009 105	+.926 ± .013 73.3	+.911 ± .015 60.2
63	+.069 ± .088 0.78	+.150 ± .087 1.73	+.311 ± .080 3.90	+.356 ± .077 4.61	+.394 ± .075 5.26	+.629 ± .053 11.8	+.819 ± .029 28.1	+.948 ± .009 105		+.994 ± .001 970	+.986 ± .002 411
70	+.065 ± .088 0.74	+.140 ± .087 1.62	+.297 ± .081 3.67	+.329 ± .079 4.17	+.350 ± .078 4.50	+.580 ± .059 9.86	+.775 ± .035 21.9	+.926 ± .013 73.3	+.994 ± .001 970		+.993 ± .001 828
77	+.053 ± .088 0.60	+.123 ± .087 1.41	+.282 ± .082 3.46	+.318 ± .080 3.99	+.333 ± .079 4.23	+.558 ± .061 9.16	+.758 ± .038 20.1	+.911 ± .015 60.2	+.986 ± .002 411	+.993 ± .001 828	

practically attained its adult size and in which there is relatively little change from one week to another.

As we follow the correlations between the later periods and preceding periods back, we note that there is a regular decrease in the values of the correlation coefficients. This may be best shown by summarizing the results graphically in diagram 1.

In the graph the correlation of the size of the organism at each

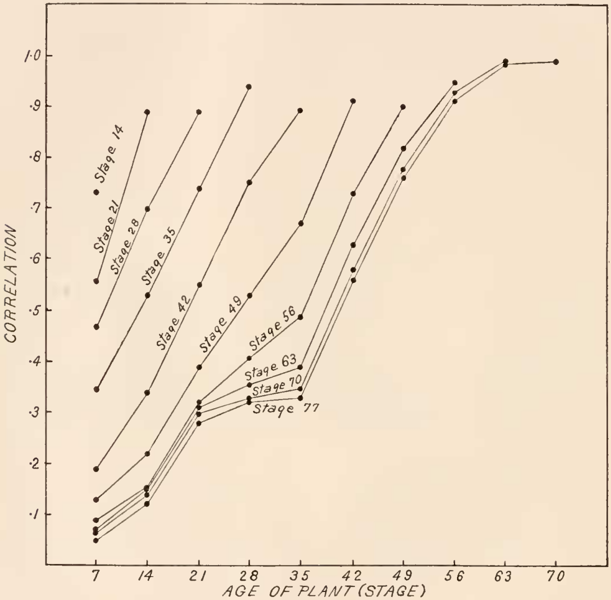


DIAGRAM 1.

growth stage with its size at every antecedent growth stage (shown at the bottom of the diagram) is shown on the scale of correlation at the left by points marking the magnitude of the correlations for each of the growth stages. The pitch of the lines connecting the points for the 14th to the 77th growth stage shows the rapid decrease in the magnitude of the correlations as the stages become more widely separated in time.

The same type of diagram may be used to show the relationship between the size at early and at later growth stages. Diagram 2 shows the distribution of the magnitudes of the correlations for sizes of the individuals at the 7th to the 70th day (stage) and the size at subsequent growth stages.

From these lines it is clear that the correlations between size

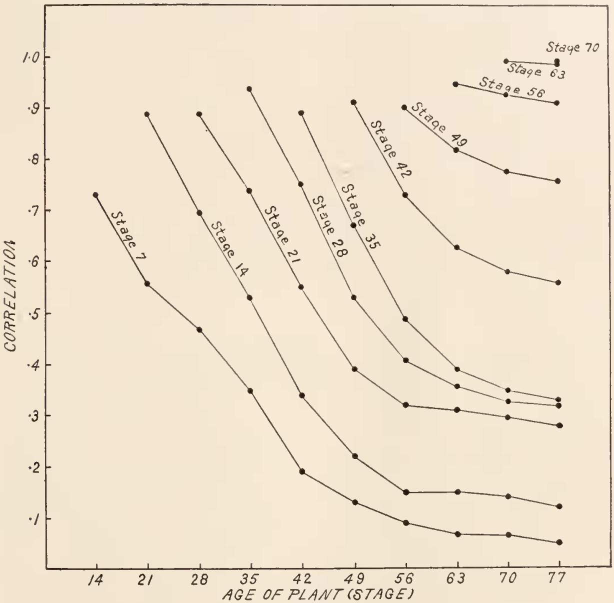


DIAGRAM 2.

at antecedent and subsequent periods decrease as the periods become more widely separated in time. This is true without exception for every period which furnishes evidence upon the question.

The coefficients are, however, positive in sign throughout, thus suggesting (though in some cases not proving) that throughout its growth period the size of the plant bears some relation to its size when first measured. This result is in agreement with the

findings of Webber (1920) in regard to the growth of *Citrus* stock.

PROBLEM 2. *The correlation between the growth increments of the organism during the several growth periods.*

Our second problem is to determine whether there is a correlation in growth increments as well as in actual size of the organism. We shall thus answer the question whether the organism which grows more rapidly than the average during one growth period will grow more rapidly than the average in other growth periods and whether the organism which lags behind the average in its rate of growth during one growth period will also lag behind during other growth periods.

Little has heretofore been done towards the statistical treatment of growth increments. This is probably in part due to the arithmetical difficulties of computing the constants for increments, but if the moments and product moments be taken about zero as origin in computing the coefficients required under Problem 1 above, the calculations for growth increments are easily made by the use of formulæ given elsewhere (Harris, 1920).

The symmetrical table showing the relationship between the actual growth increments for all of the combinations of growth periods appears as Table IV. This table shows positive and statistically significant correlation coefficients for closely associated periods throughout the season up to and including the period for the 63d to the 70th day. The coefficients for the period from the 70th to the 77th day cannot in general be considered statistically significant in comparison with their probable errors.

Examining these results in a little greater detail, we note that the nine coefficients showing the relationship between the growth increments of successive weeks (the constants bordering the diagonal cell of the symmetrical table of constants) are all positive in sign and with the exception of the last (showing the relationship between the growth of the period from the 63d to 70th and that between the 70th to 77th day) all are statistically significant. The eight coefficients measuring the correlations between the growth increments of weekly periods which are separated by one week are also without exception positive, but are lower in magnitude and less certainly statistically significant. For periods more

TABLE IV.

CORRELATIONS BETWEEN THE GROWTH INCREMENTS DURING THE SEVERAL GROWTH PERIODS.

Growth Period.	Growth Period.									
	7 to 14.	14 to 21.	21 to 28.	28 to 35.	35 to 42.	42 to 49.	49 to 56.	56 to 63.	63 to 70.	70 to 77.
7 to 14...		+.655 ± .051 12.9	+.259 ± .083 3.14	+.013 ± .089 0.14	−.115 ± .087 1.32	−.102 ± .088 1.17	−.095 ± .088 1.08	+.082 ± .088 0.93	−.070 ± .088 0.79	−.136 ± .087 1.57
14 to 21...	+.655 ± .051 12.9		+.630 ± .053 11.8	+.264 ± .082 3.21	+.064 ± .088 0.73	−.024 ± .089 0.27	+.011 ± .089 0.13	+.100 ± .088 1.14	−.082 ± .088 0.93	−.047 ± .088 0.53
21 to 28...	+.259 ± .083 3.14	+.630 ± .053 11.8		+.636 ± .053 12.1	+.161 ± .086 1.86	−.079 ± .088 0.90	−.179 ± .086 2.08	−.140 ± .087 1.61	−.249 ± .083 2.99	−.023 ± .089 0.26
28 to 35...	+.013 ± .089 0.14	+.264 ± .082 3.21	+.636 ± .053 12.1		+.532 ± .064 8.37	+.147 ± .087 1.70	−.316 ± .080 3.96	−.273 ± .082 3.33	−.483 ± .068 7.12	−.169 ± .086 1.97
35 to 42...	−.115 ± .087 1.32	+.064 ± .088 0.73	+.161 ± .086 1.86	+.532 ± .064 8.37		+.778 ± .035 22.3	+.070 ± .088 0.79	+.067 ± .088 0.76	−.189 ± .085 2.21	−.106 ± .088 1.21
42 to 49...	−.102 ± .088 1.17	−.024 ± .089 0.27	−.079 ± .088 0.90	+.147 ± .087 1.70	+.778 ± .035 22.3		+.416 ± .073 5.67	+.250 ± .083 3.01	−.005 ± .089 0.06	+.038 ± .088 0.43
49 to 56...	−.095 ± .088 1.08	+.011 ± .089 0.13	−.179 ± .086 2.08	−.316 ± .080 3.96	+.070 ± .088 0.79	+.416 ± .073 5.67		+.299 ± .081 3.70	+.453 ± .070 6.44	+.022 ± .089 0.25
56 to 63...	+.082 ± .088 0.93	+.100 ± .088 1.14	−.140 ± .087 1.61	−.273 ± .082 3.33	+.067 ± .088 0.76	+.250 ± .083 3.01	+.299 ± .081 3.70		+.476 ± .069 6.91	+.188 ± .085 2.20
63 to 70...	−.070 ± .088 0.79	−.082 ± .088 0.93	−.249 ± .083 2.99	−.483 ± .068 7.12	−.189 ± .085 2.21	−.005 ± .089 0.06	+.453 ± .070 6.44	+.476 ± .069 6.91		+.086 ± .088 0.97
70 to 77...	−.136 ± .087 1.57	−.047 ± .088 0.53	−.023 ± .089 0.26	−.169 ± .086 1.97	−.106 ± .088 1.21	+.038 ± .088 0.43	+.022 ± .089 0.25	+.188 ± .085 2.20	+.086 ± .088 0.97	

widely separated in time the correlations are in part positive and in part negative in sign.

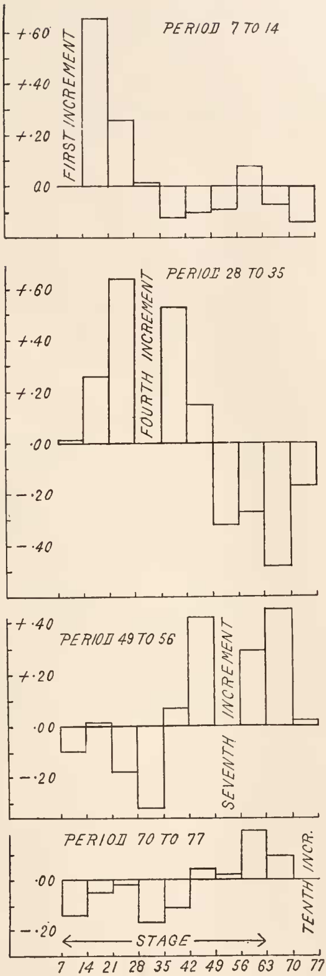
Thus from the results as a whole it appears that the increments of successive periods are generally positive and fairly highly correlated when the periods show actual growth increments. Thus the zone of coefficients lying along the diagonal cell are positive and generally fairly high. When the periods are separated by any considerable length of time the coefficients are generally insignificant in magnitude and may, as a matter of fact, be either positive or negative in sign.

The relationship may be brought out by determining the averages of the correlation coefficients, with regard to sign, for the increments of periods separated by various lengths of time. The results are as follows.

Period of Separation (Weeks).	Number of Correlations Averaged.	Average Correlation.
0	9	+.5009
1	8	+.2240
2	7	—.0334
3	6	—.1236
4	5	—.1640
5	4	—.1033
6	3	—.0077
7	2	—.0585
8	1	—.1360

If we disregard the cases in which there are less than five coefficients to be averaged, we note a steady decrease in the magnitude of the correlation coefficient. Periods of growth which are successive or separated by only one week have positively correlated growth increments. Periods which are more widely separated show negative correlations of the increments.

The relationship between the coefficients in Table IV. may be clarified by diagram 3 which shows the relationship between four of the ten growth increments and each of the other ten increments. The increments selected as a "first variable" in the correlation are the first, fourth, seventh, and tenth. This has the advantage of representing the first and the last growth increment, and of leaving undrawn no more than two successive increments. The figures are aligned according to the ten increments representing the "second variable" of the correlation.



PERIOD 7 TO 14
FIRST INCREMENT
+·60
+·40
+·20
0·0
PERIOD 28 TO 35
FOURTH INCREMENT
+·60
+·40
+·20
·00
—·20
—·40
PERIOD 49 TO 56
SEVENTH INCREMENT
+·40
+·20
·00
—·20
PERIOD 70 TO 77
TENTH INCR.
·00
—·20
STAGE
7 14 21 28 35 42 49 56 63 70 77

DIAGRAM 3.

The graphs for the first, fourth and seventh increment show clearly the shift in the position of the maximum positive correlation from the earlier to the later periods as the "first variable" is chosen from the later periods. The same is shown less clearly by the correlations for the tenth increment, but there the coefficients are very small, presumably because growth has practically ceased.

It is clear, therefore, that plants which are growing more rapidly during any period of development will grow more rapidly during a closely associated subsequent period of development but that there is little or no relationship, or even a negative relationship, between the rate of growth of the organisms studied at considerably separated periods of time.

Since the correlations for absolute growth increments are so small for all except successive periods of time, it seems unnecessary to deal at present with the relative growth increments, *i.e.*, with the growth increments expressed as a fraction of the size of the organisms at the beginning of the growth period.

PROBLEM 3. *The correlation between the absolute size of the organism at given stages of development and subsequent growth increments.*

In the higher plant organism rate of growth at any period must be supposed to depend to some extent upon plastic materials synthesized by the more nearly mature portions of the same individual. Thus one might expect to find a relationship between the actual size of the organism at any stage of growth and the rate at which the organism increases in size during a subsequent period.

We have determined the possible correlations between the absolute size of the organism at different periods and the growth increment of the organism during subsequent growth periods. The coefficients are presented in Table V. This shows positive correlation between the actual size of the organism at every stage of development from the 7th to the 70th day and the increase in the size of the organism during the following week. The magnitude of the correlation is of the order $r = 0.45$ to $r = 0.60$ for the 7th, 14th, 21st, and 28th day. For these growth stages the correlation between actual size and the subsequent growth incre-

Table V.

Correlations between Height at Each Growth Stage and the Increments at Subsequent Growth Periods.

Stage.	Growth Period.									
	7 to 14.	14 to 21.	21 to 28.	28 to 35.	35 to 42.	42 to 49.	49 to 56.	56 to 63.	63 to 70.	70 to 77.
7	+.503 ±.066 7.60	+.285 ±.081 3.50	+.239 ±.084 2.86	−.028 ±.089 0.32	−.162 ±.086 1.88	−.036 ±.088 0.41	−.046 ±.088 0.52	−.042 ±.088 0.47	−.021 ±.088 0.24	−.100 ±.088 1.14
14		+.611 ±.056 11.0	+.285 ±.081 3.50	+.000 ±.089 0.01	−.146 ±.087 1.68	−.093 ±.088 1.06	−.090 ±.088 1.03	+.050 ±.088 0.57	−.062 ±.088 0.70	−.141 ±.087 1.63
21			+.517 ±.065 7.96	+.153 ±.087 1.77	−.041 ±.088 0.46	−.063 ±.088 0.72	−.042 ±.088 0.48	+.084 ±.088 0.96	−.081 ±.088 0.92	−.103 ±.088 1.18
28				+.436 ±.072 6.08	+.062 ±.088 0.70	−.081 ±.088 0.92	−.122 ±.087 1.40	−.024 ±.089 0.27	−.183 ±.086 2.14	−.075 ±.088 0.85
35					+.255 ±.083 3.09	−.004 ±.089 0.05	−.217 ±.084 2.57	−.125 ±.087 1.43	−.329 ±.079 4.17	−.124 ±.087 1.42
42						+.360 ±.077 4.67	−.135 ±.087 1.56	−.065 ±.088 0.74	−.343 ±.078 4.39	−.145 ±.087 1.67
49							+.079 ±.088 0.89	+.059 ±.088 0.67	−.262 ±.083 3.17	−.093 ±.088 1.06
56								+.182 ±.086 2.12	−.029 ±.089 0.32	−.071 ±.088 0.81
63									+.129 ±.087 1.48	−.002 ±.089 0.03
70										+.007 ±.089 0.08

ment is clearly significant in comparison with its probable error. The coefficients are lower for the 35th and the 42d day, but are probably statistically trustworthy. Beyond this period there seems to be no relationship between the size of the organism and its growth rate in an immediately following period.

For the first two stages of growth measured, the 7th and the 14th day, there may be a significant correlation of the order $r = +.285$ between size and growth increments during the second following week.

The coefficient of correlation between size and the increment in the second week following is also positive for the 21st and 28th day, but neither of these values may be considered statistically significant in comparison with its probable error. Finally for the first stage (seventh day) there may be a significant correlation between absolute size and growth increments during the third week following ($r = +.239$ for increment for 21st to 28th day). Other than this the coefficients are for the most part statistically insignificant in comparison with their probable error.

Summarizing the preceding statements as a basis for further analysis, we note that for the first six growth stages (7th to 42d day) there is a significant positive correlation between the size of the organism at the given stage and the growth increment of the following week. For the first two growth stages (and possibly in the third where $r/E_r = 1.77$) there is a significant correlation between the size of the organism and the growth increment in the second subsequent week. Finally, for the first stage only, there is a significant positive correlation between size and growth increment in the third subsequent week.

Disregarding these 9 coefficients and the 4 positive but not significant correlations between the sizes at the several growth stages and the growth increments of the following week, we may note the following facts concerning the remaining 42 coefficients.

Of these 42 coefficients 36 are negative while only 6 are positive in sign. Of the 6 positive coefficients only that between actual size on the 21st day and growth increment between the 28th and 35th day (already considered above) is as large as its probable error. Of the 36 negative coefficients 18 are larger than

their probable error, and 5 of these are over twice as large as their probable error.

There is, therefore, clear evidence that the subsequent growth of the higher plant organism is measurably conditioned by its size. In general the larger individuals grow more rapidly in immediately subsequent periods, but somewhat more slowly than the average in more distant subsequent periods.

While a detailed discussion of the relation of these results to the theory that growth may be satisfactorily described by the curve of an autocatylic reaction falls quite outside the scope of this paper, it must be noted that negative correlations between actual size at a given stage and the growth increments of certain subsequent growth periods might be expected. As Reed and Holland (1919) have pointed out the plants attained about half their final height at about the thirty-fourth day. From this time on the increments were decreasing. Plants which had attained more than the average size at this period would, therefore, of necessity, make smaller average increase in size in later periods.

The number of individuals measured is not sufficiently large to carry the analysis farther.

III. Recapitulation.

The purpose of this paper has been to illustrate on the basis of a specific series of data the value of the inter-periodic correlation coefficient in the analysis of the phenomena of growth.

The analysis shows that in the case of a series of *Helianthus* plants the actual size of the individual at any stage of development is closely correlated with its size at other closely associated stages of development. The magnitude of the correlation rapidly diminishes as the growth stages become more widely separated in time. Thus the final size of the organism is but to a slight extent determined by its initial size.

The correlation between successive growth increments is positive in sign and statistically significant, with the general average of $\bar{r} = .501$. The correlation for increments of weekly periods separated by one week is on the average only about $\bar{r} = .225$. For periods more widely separated than this the correlation between growth increments is on the average negative in sign.

Thus plants which are growing more rapidly during one period of development will grow more rapidly during a closely associated period, but there is little or no relationship between the growth increments of more widely separated periods.

The growth increment of the organism is positively correlated with its size at an immediately preceding stage. In the early stages of growth, the growth increments of two or even three subsequent periods are positively correlated with the initial size of the organism.

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THE CHROMOSOMES OF PSEUDOCOCCUS NIPÆ.

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Introduction.

In the course of some work on sex determination in the different species of *Pseudococcus*—a genus of the Homoptera—very peculiar conditions were met with in the chromosome behavior. These peculiarities were observed especially in *Pseudococcus nipæ* (identified by H. Morrison, Bureau of Entomology), and it is of this species that the present account is given. A more detailed report, covering other species of *Pseudococcus* as well, is reserved for a later paper.

Most of the material was fixed in Allen's modification of Bouin's fluid. On the whole, fixation is more or less difficult; and at best the cells are somewhat small. The main features are clear cut, however, and hardly to be mistaken. I am indebted to Professor E. B. Wilson and to Professor C. E. McClung who examined some of my slides and offered helpful criticism.

The Chromosomes in the Female.

The number of somatic chromosomes in the female is ten, with little or no size and form differentiation. Counts are made with little difficulty in various cells, but oögonial cells furnish of course the best criterion. Generally the chromosomes are counted most easily just before they have become arranged in a metaphase plate. There can be no doubt as to their number (Fig. 6). A detailed study of the maturation phenomena in the female was not made. Suffice it to say that five tetrads are formed and that these are normal in appearance; they are very much like those formed in the oögenesis of many other Homoptera (Fig. 7). The reduction process is thus probably not unusual.

Spermatogenesis.

The somatic number of chromosomes in the male is also ten, and as in the female these seem to be alike in size and shape.

Such chromosome counts were generally made in cells of the developing nervous tissue where division phases are common (Figs. 3 and 4).

Spermatogonial divisions seem to be completed with comparative speed, for specimens which show them are not plentiful. Just as in the somatic cells the number here is undoubtedly ten (Fig. 5).

The stage following the spermatogonia seems to be much longer in duration. The cells increase perceptibly in size during this time. The earliest phase observable shows some flocculent masses of lightly staining chromatin irregularly distributed through the nucleus. At one point, always at the periphery of the nucleus, there is a more deeply staining mass. Nothing concerning the structure of this can be made out and its shape is variable (Fig. 8). With progressive development this deeply staining mass undergoes a few, very definite, changes. In successive steps it appears that a number of more or less irregular lumps is evolved. Still massed at first, these gradually become separated and then it is certain that they are five in number (Figs. 8 to 11). It is at this latter stage that a split is occasionally visible in some of them, but with increasing condensation this again becomes obliterated. Throughout this development these five bodies retain a definite tendency to remain in close proximity to each other, and this tendency is one that persists also through subsequent stages.

In the meanwhile the flocculent and more lightly staining chromatin has also undergone development. Before the denser mass has become evolved into five distinct bodies, this chromatin has been transformed into a fine network of threads. Apparently these are polarized toward the dense mass (Fig. 9). Like the leptotene threads of other forms, these threads shorten and thicken, a process accompanied by a progressive increase of their staining intensity. Polarization is finally lost, and already at this stage it becomes apparent that the number of shortened threads is less than ten (Fig. 10). As the threads continue their process of shortening, they are counted with greater ease, and in such a stage as shown in Fig. 11 it becomes certain that they are five in number. Like the denser bodies, these sometimes show a longitudinal split.

Both the denser bodies as well as the threads continue progressive condensation, and the former reach their final form some time ahead of the latter (Fig. 12). They are then somewhat oblong in shape, and take the hæmatoxylin stain with great intensity and there can be no doubt of their chromosomal nature. Somewhat later the erstwhile threads have also assumed this form, and there are then ten of these bodies or chromosomes, identical in size and shape. Those first evolved continue to betray a certain affinity for each other, and in the metaphase plate constitute a central group around which the other five chromosomes become ranged in no definite order (Figs. 13 and 14). Aside from this very characteristic grouping, the only difference between the two sets of chromosomes that is apparent consists in the rate at which they evolve or the stage which is the starting point of their development.

Throughout this development, there has been no trace of a tetrad formation. The general features of the case indicate that the split which was spoken of as occurring at one stage is nothing more than preparation for the equation division or else something of the nature of the "Querkerbe" observable in lower Crustacea.

Division now occurs in ordinary manner and ten chromosomes go to each pole (Figs. 15 to 17). The arrangement of chromosomes in the daughter cells is not absolutely certain, although fig. 16 indicates that there also the characteristic grouping is retained. Figures like these are too rare to admit of any definite conclusion however. At any rate, the time in which such an arrangement persists must be very short, for the chromosomes are generally found in a more or less irregular heap (Fig. 18).

The division just described is undoubtedly equational in character. Following it there seems to be no intervening further development in the chromosomes of the daughter cells. Instead, they begin to scatter in a longitudinal direction. This process is not entirely irregular however for it results in their separation into two groups of five each (Figs. 18 to 22). It is a remarkable feature that these two groups are each characterized by a distinct and different arrangement of their component chromosomes. The group going to one pole assumes the form of a V or a

triangle, while the sister group which goes to the opposite pole is circular or lumped in arrangement (Figs. 21 and 22). This grouping is so constant and has been observed in so many specimens, that no mistake seems possible, and the conclusion seems inescapable that it is of some significance. The telophase of this anomalous division still shows traces of the arrangement, but these are soon lost as the chromosomes of each daughter cell distribute themselves around the periphery of the nucleus. Their number here is undoubtedly five (Fig. 24). This initiates the formation of the spermatids in which the chromosomes gradually loose their staining reaction. No study of the subsequent stages was made except to determine that there is no sign of degenerating or abortive cells nor a size dimorphism in the spermatozoa.

Somatic Chromosomes.

Returning to the somatic tissues, it may be remarked here that although the number of chromosomes in each sex is the same, their arrangement differs in the two sexes. This is especially noticeable in the developing nerve tract, where in the male the cells in the resting stage show a relatively large nucleolus like structure (Fig. 2). This is not to be seen in the same tissue in the female where cells show only the flocculent chromatin peculiar to that phase (Fig. 1). That the nucleolus-like structure in the male nerve cells is nothing but the group of five chromosomes mentioned in the description of the spermatogenesis becomes almost certain in metaphase plates found in the same tissue. Figs. 3 and 4 show such grouping without a doubt.

Exactly the same feature is observable in spermatogonial plates, though the size of these renders them less favorable (Fig. 5). In contradistinction, oogonial plates have no such arrangement, and even in such a late stage before division as shown in Fig. 6 the chromosomes are arranged in no definite order.

Discussion.

An interpretation of these observations is perhaps not out of place. It is here given with the idea that it should not affect the observations however it may be received and is advanced in a speculative way.

As has already been stated, oögenesis very probably follows ordinary lines. The ten chromosomes constituting the diploid number are composed of five homologous pairs, and these synapse and form tetrads. Reduction is very probably normal, and results in a pronucleus with five chromosomes.

In the spermatogenesis, the spermatogonial divisions, like the somatic divisions, also occur in orthodox manner. This is apparently not true of the meiotic divisions however. In explanation of these, the best hypothesis is one which views the various developments in the light of sex chromosomal behavior and is as follows:

The central group of chromosomes which appears in the growth stages of the male as the more densely staining mass contains sex chromatin, equally distributed among the five chromosomes. The remaining chromosomes, which stain lightly at first, represent what may be regarded as purely autosomal chromatin. Granting this, and the assumption does not appear unjust in the light of what has been described, the seemingly peculiar development becomes a natural consequence. Just as in the spermatogenesis of the various Orthoptera and Hemiptera, the sex chromosomes always stain more or less intensely, and as far as observable do not go through the various stages of thread formation. That such formation may occur earlier, or in a restricted sense even while the dense mass is still irregular in outline, is not ruled out by any means. The autosomal chromatin on the other hand goes through all the usual steps, culminating in the formation of five chromosomes. The sex chromatin contained in the five grouped chromosomes will tend to explain their grouped arrangement, since again as in the Hemiptera, multiple X or X and Y chromosomes show a tendency to remain in close proximity during development.

If now the sex chromosomes in *Pseudococcus nipæ* are regarded homologous in every way to the autosomes, except that each carries a certain amount of sex chromatin, the subsequent behavior is just as would be expected. I may mention here that such sex chromosomes would imply a more intimate union of sex and autosomal chromatin than is illustrated by such a case as

Mermiria (McClung, '05) where the sex chromosome and the autosomes are distinct, but the former is attached to one of the latter. It is at present unnecessary to go into the relation of the two conditions, though very possibly they represent two distinct steps in the phylogeny of sex chromosomes.

Their subsequent behavior is more or less analogous to that of the X Y pair in other forms. This pair does not form a tetrad in the ordinary sense simply because its members are not homologous, or better perhaps, because neither has a true synaptic mate. When as in the homozygous state both members of a pair of sex chromosomes are homologous, synapsis and tetrad formation occur just as in the autosomes. This fact is plainly borne out in the oögenesis of many Hemiptera (Morrill, '10) as well as in the growth and maturation phenomena of the eggs of *Pseudococcus nipæ*. It is thus to be assumed that if in the present case of the spermatogenesis of *Pseudococcus nipæ* the sex chromatin were distributed equally over the ten autosomes, the pairs would be homologous and tetrads would be formed in the usual way.

The cytological evidence indicates nothing that should render an equation division exceptional in nature, and it does indeed occur in the usual manner. The second division witnesses reduction in that the autosomes carrying sex chromatin go to one pole while the purely autosomal chromosomes go to the opposite pole. Taking recourse to a parallel case once more, attention may be drawn to the two X chromosomes in *Syromastes* which always go the same pole in reduction (Wilson, '09). Similarly, the multiple X of the Reduviidæ always goes to one pole, although this is not an exactly parallel case since it is probably the product of fragmentation of a single X.

Thus to repeat what has already been intimated for the present case, the distribution of the chromosomes to their respective poles in the reduction division may be explained on the ground that we are concerned with five pairs of chromosomes. The members of each pair are homologous except for the fact that one of them in each instance carries a certain amount of sex chromatin. The presence of the latter does not influence the behavior of the chromosome pairs in reduction and the members of each pair go

to opposite poles. Its presence does however prevent haphazard distribution in that the five chromosomes carrying this sex chromation tend to remain clustered or grouped and therefore go to the same pole.

Although more or less contrary to the cytological evidence furnished by other groups of insects, it may not be amiss to suggest the possibility that in animals with haploid males each chromosome carries a certain amount of sex chromatin. It follows that the diploid female would then represent 2 X, whereas the haploid male would represent 1 X. In the haploid male the reduction division is not truly abortional as has been supposed, but is merely a division in which these sex chromatin carrying chromosomes go to one pole while the opposite pole receives no chromosomes simply because the mates to these chromosomes are absent. It is of interest to note that the straggling or lagging so often observed in the sex chromosomes of various insects is paralleled by the scattered and irregular distribution of the chromosomes on the spindle of this division in the Hymenoptera. And lastly, such irregular distribution is found also in the reduction division of *Pseudococcus nipæ*.

Pseudococcus nipæ thus would stand half way between forms with haploid males where every chromosome carries sex chromatin, and forms in which the sex chromatin is carried in very few chromosomes and there is little numerical variation in the chromosomes of the two sexes. In other words, half of the chromosomes in the males carry sex chromatin.

Although superficially an instance of Weismann's postulated ideal type of reduction in which the diploid number of chromosomes is halved without previous syndesis, the spermatogenesis of *Pseudococcus nipæ* nevertheless follows the commonly accepted lines of meiosis. The apparently exceptional behavior can be explained as due to an extreme mode of sex chromatin distribution and is not a unique example of the *Primærtypus* of reduction. It may be remembered that Goldschmidt ('05 and '08) gave this name to an instance of Weismann's simple type which he thought to have discovered in *Zoögonus mirus*. The Schreiners ('08) examining Goldschmidts slides believed to have

found a serious error in his counts of somatic chromosomes, which they believed in reality to be 24 and not 10 as he had reported. Furthermore, reduction occurred in the ordinary way, just as in *Tomopteris*. Gregoire ('09) in going over the same slides maintained that the Schreiners were correct in that the case was one of ordinary reduction, but that they in turn had made an error in the chromosome counts. The somatic number is about 12, and the reduced number 6. Lastly Wassermann ('11, '12, and '13) procured new material and concluded that Gregoire's counts had been correct. He did not agree with Gregoire as to the mode of synapsis however, and apparently was unable to reach a final conclusion in this regard himself. Although he thus does not believe that the question has received a definite settlement, the fact remains that Zoögonus does not represent the simple type of reduction that Weismann advanced in a hypothetical way.

If my hypothesis is correct, the male of *Pseudococcus nipæ* is heterozygous in that it has five sex-chromatin carrying chromosomes and five chromosomes purely autosomal in character. Crossing over would not occur in these chromosomes. It would occur however in the female, in which the ten chromosomes are composed of five homologous pairs. If the male represents 1 X, the female with ten sex-chromatin carrying chromosomes represents 2 X.

Summary.

1. The diploid number of chromosomes in *Pseudococcus nipæ* is ten in both sexes.

2. In the maturation of the egg, five tetrads are formed and reduction is probably normal.

3. In the spermatogenesis, five chromosomes are developed before the others, and these tend to remain grouped together.

4. No tetrads are formed, and in reduction five chromosomes go to one pole (supposedly those evolved first) and five to the other.

5. Explanation of this seemingly anomalous behavior is to be sought in the fact that five of the chromosomes carry sex chromatin.

6. The case is not so much to be regarded as an illustration of Weismann's ideal type of reduction, as an exceptional example of reduction due to unusual sex chromatin distribution.

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All drawings made with a Spencer 15 eyepiece and Zeiss 1.5 objective with the exception of Figs. 1 and 2, where a 10 eyepiece and 2 mm. objective were used.

PLATE I.

1. Cells in nervous tissue of the female.
2. Cells in nervous tissue of the male.
3 and 4. Metaphase plates from nerve tissue of the male.
5. Spermatogonial plates.
6. Oögonial cell.
7. Tetrads prior to polar body formation in the egg.
8 to 12. Growth stages prior to first division.
13. Metaphase plate of first division.

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Plate II.

14. Metaphase plate of first division.
15. Side view of plate.
16 to 18. First division (equational).
19 to 23. Second or reduction disvision
24 and 25. Spermatids.


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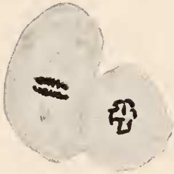
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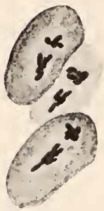
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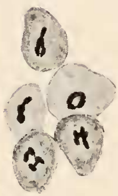
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AUTHOR'S ABSTRACT OF THIS PAPER ISSUED BY
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OBSERVATIONS ON THE LARVÆ OF CORETHRA PUNCTIPENNIS SAY.

CHANCEY JUDAY.[1]

Introduction.

As part of a general problem relating to the biological productivity of lakes, a quantitative survey of the bottom fauna in the deeper portions of Lake Mendota at Madison, Wisconsin, was made between the early part of May, 1916, and the middle of August, 1918. This survey included only the macroscopic forms, such as the insect larvæ, the Oligochæta, and the Mollusca. The investigation showed that the full-grown larvæ of *Corethra punctipennis* Say constitute the principal element of the bottom population in the daytime during the greater part of the year. For at least three quarters of the year, in fact, they not only far outnumber all of the other forms combined, but they also exceed them in total weight. The great abundance of these larvæ thus makes them a very important factor in the biological complex of the lake.

Samples of the bottom were obtained by means of a modified form of the Ekman dredge; the opening of this instrument covered an area of 473 square centimeters. The mud obtained in each haul of the dredge was washed through a gauze net having meshes fine enough to retain all of the macroscopic forms. The material secured by the net was transferred to a jar and was then taken to the laboratory where the various organisms were sorted out and enumerated in the living state. The average live and dry weights of the various forms were also obtained, as well as the percentage of ash.

Observations were made at five regular stations located in water having a depth of 20.5 meters to 23.5 meters. These stations were widely separated in order to secure a fair average of the density of the bottom population in the deeper portions of the

[1] Notes from the Biological Laboratory of the Wisconsin Geological and Natural History Survey No. XXI.

lake. The results obtained at only one of these stations, designated as Station II., are considered here, however, because the other four were not visited regularly during the winter months. From April to June inclusive, the number of larvæ found at Station II. was from 10 per cent. to 20 per cent. larger than the general average of the five stations during these months, but in August the general average was larger than the numbers at Station II. In the six sets of averages obtained for the last three months of the year in 1916 and 1917, three of the averages for Station II. were larger than the corresponding averages of the five stations and three were smaller. Thus, the numbers obtained at Station II. were somewhat larger than the general average for the deeper part of the lake as a whole during the first half of the year, but they were somewhat smaller from July to September and substantially the same from October to December.

Numerical Results.

The average number of *Corethra* larvæ per square meter of bottom is shown for the different months of the year in Table I. Large numbers of these larvæ live over winter; in fact they are

Table I.

The Number of Corethra Larvæ per Square Meter Obtained from the Mud in the Daytime at Station II. during the Different Months of the Year.

In all months except January and February the numbers represent averages of two to nine samples.

Year.	January.	February.	March.	April.	May.	June.
1916.....					17,300	4,550
1917.....	25,340	23,740	30,380	23,230	17,900	16,000
1918.....		23,440	20,160	18,940	18,170	9,430
Year.	**July.**	**August.**	**September.**	**October.**	**November.**	**December.**
1916.....	3,380		7,400	17,600	26,900	26,900
1917.....	6,080	800	3,740	11,500	17,700	24,800
1918.....	2,820	960				

more numerous from November to April than at any other time of the year. During this interval the numbers range from approximately 18,000 to 30,000 individuals per square meter. At

this time of the year there is no loss from pupation and the losses from other causes are not great enough to reduce the number of these larvæ very materially, so that the number remains uniformly high during this period of time.

The ice usually disappears from the lake during the first week in April and soon after this event the larvæ begin to pupate. As the temperature of the water rises, the rate of pupation increases so that an appreciable decrease in the number of larvæ is noted for the month of May. With the further advance of the season, pupation becomes still more common and this results in a very marked decrease in the number of *Corethra* larvæ in late June as in 1916 and in 1918, or in early July as in 1917. This decline in numbers continues until the minimum of the year is reached in August, more especially during the first half of this month. A minimum of 295 larvæ per square meter was noted at Station II. on August 2, 1917, while the average in March of this year was a little more than one hundred times as large. (See Table I.)

Small swarms of adults appear in May and in early June, but the great flights are correlated in time with waves of very active pupation in late June, in July, and in early August. Thus, enormous swarms appear from time to time during the latter period.

During late August and especially in September there is a slackening in the process of pupation and correlated with this is an increase in the number of larvæ. The increase is most marked during the second half of September and in early October, but the numbers do not reach the maximum point until November or December. The largest number of larvæ obtained in any of the samples was 33,800 individuals per square meter on December 21, 1917.

Pupæ were not noted in the samples of mud until about the middle of June, or at the beginning of the more active period of pupation; thereafter they appeared regularly until late August. A maximum of 2,890 pupæ per square meter was found on June 28, 1917, while the second in rank was 1,370 individuals per square meter on July 9, 1917.

Deposition and Development of Eggs.

According to Muttkowski the adults emerge at night, "beginning early in the evening and continuing through the night. In the morning, if the lake is quiet, the females can be seen on the surface, ovipositing through the surface film." Eggs deposited by females kept in insect cages sink to the bottom of the aquarium and experimental evidence indicates that those deposited at the surface of the lake also sink to the bottom. Mud from Station II. where the water is 23.5 meters deep and also from Station I., located in water 18.5 meters deep, was washed through a sieve with meshes fine enough to remove all of the larvæ; this sifted mud was then placed in aquaria. At the end of five days a dozen small *Corethra* larvæ had appeared in the material from Station II., while five small larvæ were noted in the other bottom material at the end of a week, thus showing that the mud from both stations contained eggs.

Eggs that were deposited by females kept in captivity hatched within forty-eight hours when the temperature of the laboratory ranged from 21° to 24° C. The temperature of the lower water in the deeper portions of the lake is much lower than this, however, and the eggs which reach the bottom in these areas probably do not develop so rapidly. The bottom temperature at Station II. in summer, for example, ranges from slightly more than 9° in some years to about 14° in other years. On the other hand, eggs deposited in water not exceeding five meters in depth are subject to temperatures of 20° to 25° in July and August so that they probably hatch about as promptly as those kept under laboratory conditions.

Another factor that may retard development in the deeper water is the absence of free oxygen. Usually all of the dissolved oxygen below a depth of 18 meters is used up by the middle of July, after which no oxygen is available in this region until October. Thus, all of the *Corethra* eggs which reach the bottom in water that is 18 meters deep or more during this period must develop under anaërobic conditions if they develop at all. This anaërobic stage covers the greater part of the most active reproductive period of this insect and approximately 30 per cent. of

the area of the lake lies within the 18 meter contour. For two or three weeks in August, in fact, a little more than half of the lake bottom is subject to anaërobic conditions. No attempt was made to ascertain the effect of this lack of oxygen on the development of the *Corethra* eggs. Many cocoons of the Oligochæt *Limnodrilus* were noted in the bottom material during this period, however, and the eggs in them seemed to be developing normally in the absence of oxygen. This fact suggests that the eggs of *Corethra* may also develop normally under anaërobic conditions.

The young *Corethra* larvæ were not noted in the series of net catches until the last week in June, though the eggs of the first adults each year undoubtedly hatch at an earlier date than this. They were found regularly in the net catches from the latter part of June to the first week in October.

Behavior.

In 1917 and 1918 net hauls were made regularly at three of the stations before the mud catch was taken in order to see if any of the full-grown larvæ occupied the water in the daytime, but the results were entirely negative. Some of these hauls were made as early as 8:30 A.M. and others as late as 4:30 P.M., so that these observations covered the chief portion of the day. It was found also that the full-grown larvæ deserted the water on cloudy days as well as on clear days.

A series of observations made at Station II. during the afternoon and evening of July 16, 1917, showed that the full-grown *Corethra* larvæ had not emerged from the mud by 7:30 P.M., or just about sunset. At 8:00 P.M., or half an hour after sunset, 133 larvæ and 88 pupæ per square meter of lake surface were found in the water. By 8:30 P.M., or one hour after sunset, these numbers had increased to 3,945 larvæ and 442 pupæ. At the latter hour the full-grown larvæ had reached the surface of the lake, thus showing a vertical migration of 23.5 meters during an interval of about one hour.

A similar set of observations was made in 1920 beginning at 5:45 P.M. on June 10 and continuing until 5:30 A.M. on June 11. On the former date sunset came at 7:36 P.M., standard time, and sunrise on the following day at 4:18 A.M. No *Co-*

rethra larvæ were obtained from the water on June 10 until 7:15 P.M., at which time a catch yielded 22 individuals per square meter of lake surface. Fifteen minutes later, or just a few minutes before sunset, this number had risen to 176 larvæ per square meter, and by 7:50 P.M., or about a quarter of an hour after sunset, the number was 1,576. At 7:36 P.M. the larvæ had not invaded the upper 10 meters of water, but they had ascended to the 10–15 meter stratum. They did not appear at the surface until about an hour and a quarter after sunset, so that the rate of upward migration was somewhat slower than that noted in 1917. Pupæ reached the surface at 9:00 P.M., or approximately an hour and a half after sunset. The largest number of both larvæ and pupæ found in the water during this set of observations was noted in a catch taken at 10:00 P.M.; of the former there were 4,730 individuals per square meter of lake surface and of the latter 287. By 11:00 P.M. the numbers had declined to 2,100 and 110 respectively; the numbers were substantially the same as these at 2:00 A.M. on June 11.

Larvæ were still found in the upper meter of water at 3:30 A.M., but they had disappeared from the upper 10 meters by 3:47 A.M. and only one individual was obtained in a catch taken from the 0–15 meter stratum at 3:50 A.M. Practically, then, they deserted the upper 15 meters of water during a period of about 20 minutes. It should be noted, also, that this downward migration was not due to direct sunlight since it took place at least half an hour before sunrise. The larvæ were still occupying the 15–23 meter stratum in considerable numbers, since a catch at 3:55 A.M. yielded 1,658 individuals per square meter in that region; the same catch contained 88 pupæ per square meter also. The number of larvæ in the lower water then gradually diminished, the last disappearing between 4:45 and 5:00 A.M. According to these results, then, the full-grown *Corethra* larvæ enter the bottom mud by the end of the first half hour after sunrise and they remain there until about sunset.

Samples of mud taken at 6:00 and at 7:00 P.M. on June 10, 1920, yielded an average of 2,720 larvæ and 55 pupæ per square meter of bottom; as a result of the migration into the water these numbers had declined to 1,400 larvæ and 22 pupæ per square

meter at 8:00 P.M., while the samples obtained during the next three hours yielded from 1,600 to 2,100 larvæ. The latter number was also found at 3:00 A.M., but it rose to 2,665 at 4:00 A.M. and to slightly more than 3,000 per square meter at 5:00 A.M. Thus the mud contained from one half to two thirds as many larvæ at night as were found there in the daytime.

For a certain period after they hatch out, the behavior of the young larvæ is very different in the daytime from that of the full-grown individuals; that is, the former occupy the lower water during the daylight hours instead of the mud, being found in the lower part of the mesolimnion and in the hypolimnion. The young larvæ migrate into the upper water at night just as the full-grown ones do. It has not been definitely determined just how long this difference in behavior lasts; only rarely was an individual found in the mud which was estimated to be only one third as large as a full-grown larva and frequently individuals were obtained from the water which were recorded as half grown. Thus, it appears that the young larvæ inhabit the lower water in the daytime instead of the mud until they are approximately one third grown, or perhaps a little larger. Muttkowski states that the larval period lasts from six to seven weeks in the summer broods; on this basis it may be estimated that the difference in behavior between the young and full-grown larvæ continues for the first ten days or two weeks of the larval period.

A series of catches was made with a plankton trap on August 2, 1917, for the purpose of ascertaining the vertical distribution of the small larvæ. The results are shown in Table II. No larvæ were found in the upper 8 meters, but they appeared at 10 meters and at greater depths. The maximum number, 489 individuals per cubic meter of water, was obtained at a depth of 18 meters, which was about the middle of the hypolimnion. Somewhat more than 88 per cent. of the total number of individuals occupied the 15–20 meter stratum.

Some results obtained on Devils Lake, Wisconsin, show that the behavior of the full-grown larvæ of *Corethra plumicornis* Fabricius[1] differs in the daytime from that of *C. punctipennis* in

[1] Dr. J. R. Malloch kindly identified this larva.

TABLE II.

THE NUMBER OF YOUNG CORETHRA LARVÆ PER CUBIC METER OF WATER AT DIFFERENT DEPTHS OF LAKE MENDOTA ON AUGUST 2, 1917.

Those obtained at 10 meters and 12 meters were recorded as very small and for the other depths the individuals were estimated to be from a quarter to a third as large as full grown larvæ.

Depth in Meters.	Temperature, Degrees C.	Number of Larvæ per Cubic Meter.
8	19.8	0
10	17.4	44
12	16.0	67
15	14.5	200
18	13.6	489
20	13.5	311
23	13.3	22

Lake Mendota. In the former lake two net catches on May 25, 1917, which were made in the deepest water, namely, 14 meters, gave an average of 422 full-grown *C. plumicornis* larvæ per square meter of surface, while two hauls of mud at the same place yielded an average of 433 individuals per square meter. That is, these larvæ were substantially equally divided between the water and the mud at about 10:00 A.M. on a bright morning when the water was so transparent that a white disc 10 centimeters in diameter did not disappear from view until it reached a depth of 8.6 meters. In other words, the day distribution of the larvæ of *C. plumicornis* was practically the same in Devils Lake as the nocturnal distribution of the larvæ of *C. punctipennis* in Lake Mendota.

While the larvæ of *Corethra punctipennis* give a prompt negative reaction to light, yet it hardly seems probable that their extensive depth migration in Lake Mendota, even including a descent into the mud, is a simple light phenomenon. The transparency of the water is usually low in summer; a white disc 10 centimeters in diameter generally disappears from view at a depth of two meters to about four meters at this season of the year, which indicates that the light is cut off rather rapidly by the upper strata of water. On the morning of June 11, 1920, for example, the disc reading was 4.25 meters. A pyrlimnimeter has been used to determine the rate at which the sun's energy is cut off by the upper strata of the lake. The results obtained with this instru-

ment indicate that the intensity of the illumination at a depth of 23 meters on a clear day, between 11:00 A.M. and 1:00 P.M., is about equal to that produced by full moonlight at the surface of the lake. During the early forenoon and the late afternoon, as well as on cloudy days, the illumination is much smaller than this. For some time before sunset, the bottom stratum must be substantially in total darkness, yet the observations show that the emergence of the larvæ from the mud is very closely correlated in time with the setting of the sun.

Not only does the illumination in the bottom water become very small in the late afternoon, but there is a further protection from light afforded by the bottom ooze in which the larvæ remain concealed during the day. To what depth the larvæ penetrate the loose mud is not known, but in the laboratory they readily burrow down to a depth of a centimeter or more. The dim light which reaches the bottom in the deeper portions of the lake can penetrate the ooze only to a very slight extent at most, even during the brightest part of the day, and this raises the very interesting question as to what stimulus causes the larvæ and pupæ to emerge from the mud so promptly and regularly about the time of sunset. No definite data bearing on this point have yet been obtained.

These larvæ are eaten with avidity by many fishes and their habit of occupying the mud in the daytime may thus serve a very important purpose from the standpoint of protection from such enemies. A further protection is afforded by the disappearance of the dissolved oxygen in the hypolimnion. Usually by the first of August very little free oxygen remains in this stratum, which makes the lower water unfit for the permanent occupation of the larger forms which prey upon these larvæ. In spite of the lack of oxygen, however, Pearse and Achtenberg found that the yellow perch—*Perca flavescens* (Mitchill)—invades the lower water and feeds upon these larvæ. While these fish survive for a period of two hours in water that contains no dissolved oxygen, these authors state that it is doubtful whether a perch is able to feed for more than a few minutes at a time under such conditions. It seems probable, therefore, that the *Corethra* larvæ are not eaten as freely as they might be if anaërobic conditions did not

prevail in the lower strata of the deeper water. Also, the absence of dissolved oxygen in the hypolimnion serves as a protection to the young larvæ which occupy this region in the daytime for a certain period after they hatch out.

Number in Shallower Water.

The larvæ of *Corethra punctipennis* show a decided preference for the deepest portion of Lake Mendota. In the daytime, they are much more abundant in the mud where the water reaches a depth of 20 meters or more than they are in the shallower areas. It was found that the average number of larvæ within the area bounded by the 20 meter contour line was more than three times as large as the average for the region lying between the 8 meter and the 20 meter contours, while the number obtained in areas where the water did not exceed five meters in depth was practically negligible.

Some three hundred samples were taken in series which extended from the shallow water to the deep water; that is, from a depth of 8 meters or 10 meters down to a depth of 20 meters. The results of four sets of these observations are shown in Table III. It will be noted that there was a marked increase in the

Table III.

The Number of Corethra Larvæ per Square Meter of Bottom at Different Depths in Four Sets of Observations which were Made in 1917.

Date.	Depth in Meters.	Number.	Date.	Depth in Meters.	Number.
May 15	10.5	820	September 24	10	110
	12.5	1,600		12	250
	15.5	4,640		15	5,500
	18	4,810		18	7,490
	20	7,800		20	13,380
June 22	10	100	October 24	12	85
	12	85		15	2,740
	15	1,080		18	11,650
	18.5	1,710		20	15,930
	20.5	3,610			

number of larvæ correlated with the increase in the depth of the water. On May 15, for example, the sample taken at 15 meters yielded about six times as many as the one at 10 meters, while

that at 20 meters gave nearly ten times as many as the latter. On September 24, the differences were fiftyfold and more than a hundredfold, respectively, and on October 24 the number was nearly two hundred times as large at 20 meters as at 12 meters.

Just how these larvæ are able to constantly maintain such a marked difference in numbers in favor of the deep water is a puzzling question. Their method of locomotion would not lead one to expect them to travel very far of their own accord should they reach the shallow areas, yet it seems probable that many of them are carried into the shallow water by the currents when they migrate into the upper strata at night. This would be true especially on windy nights. Table III. shows that a very small number of larvæ is found at 10 meters as compared with the deep water and 39 per cent. of the area of the lake lies outside the 10 meter contour line. The number is usually not much larger at 12 meters than at 10 meters and the former divides the area of the lake approximately into halves. The outer or shallower half of the lake, then, is very sparsely populated by these larvæ, but it is not clear just how the number is kept so small in comparison with the inner or deeper half of the lake.

In a large proportion of the former area the bottom does not consist of material in which the larvæ can readily conceal themselves in the daytime, being composed of sand, gravel, and rock, so that the tendency would be to avoid these areas. On the other hand, the larvæ are no more abundant in the shallow portions of protected bays where a muddy bottom suitable for concealment is found at a depth of only 5 meters or 6 meters. The difference can scarcely be attributed to a proportionally unequal distribution of eggs between the two regions because very large numbers of egg bearing females are found over the shallow water as well as along the shore; it seems probable, therefore, that enormous numbers of eggs are deposited in the shallow areas.

When the larvæ migrate into the upper strata of the lake at night, the direct currents tend to carry them into the shallow water, but the return currents, on the other hand, will aid more or less in bringing them back to the deep water. Apparently the chief factors governing the distribution of the *Corethra* larvæ

between the shallow half and the deep half of the lake are (1) an active migration, (2) the currents—direct currents on the windward side of the lake and return currents on the leeward side, (3) a relatively greater loss in the shallow water due to predatory enemies.

Number in Other Lakes.

For purposes of comparison similar quantitative studies of the bottom population were made regularly in Lake Monona and in Lake Waubesa during 1917. The former is only one kilometer from Lake Mendota and has nearly as great a maximum depth, namely, 22.5 meters. Lake Waubesa lies about seven kilometers southeast of Lake Mendota, but it is a much shallower body of water, having a maximum depth of only a little more than 11 meters. In Lake Monona only about one tenth as many *Corethra* larvæ were found as at corresponding depths and times in Lake Mendota; in some instances the difference was more than a hundredfold in favor of the latter lake. In the deepest part of Lake Waubesa the number varied from about the same as that at 11 meters in Lake Mendota to only a third or a quarter as many; but the deeper water of Lake Mendota yielded from forty to a hundred times as many larve as the deepest portion of Lake Waubesa. Bottom material has been obtained from about a dozen other Wisconsin lakes and in all of them the *Corethra* population has been relatively small, which seems to indicate that Lake Mendota offers a particularly favorable habitat for these larvæ.

Gravimetric Results.

Between June, 1916, and April, 1917, more than fourteen thousand larvæ of *Corethra punctipennis* were picked out of the material collected in Lake Mendota and they were dried for the purpose of making a chemical analysis of them. The average amount of dry matter per individual for this number was 0.251 milligram. The average weight was also determined for eleven other lots of larvæ containing from 100 to 300 individuals each. These averages ranged from a maximum of 0.311 milligram per larva, dry matter, in June to a minimum of 0.182 milligram in early September. The results of these weighings are shown in

Table IV. The higher average of dry matter in the June material may be due to a larger proportion of chitin in the larvæ just before they pupate. In August and early September the average size of the larvæ seems to be smaller than that of the winter brood and this is confirmed by the weights. At the height of the pupating season the summer larvæ pupate when they are distinctly smaller than the individuals which live over winter. The live weights of the smaller lots were also determined, as shown in Table IV., and they indicate that about 91 per cent. of the living animal consists of water.

The live weight of the June pupæ was only about 11 per cent. larger than that of the June larvæ, but the dry weight of the former was nearly twice as large as that of the latter. (See Table IV.) This marked difference in the dry weight was probably due to the presence of a larger amount of chitin in the pupa.

TABLE IV.

THE AVERAGE WEIGHT OF A SINGLE INDIVIDUAL OF CORETHRA PUNCTIPENNIS IN MILLIGRAMS, TOGETHER WITH THE PERCENTAGES OF WATER AND OF ASH.

Form.	Month.	Live Weight in Milligrams.	Dry Weight in Milligrams.	Per Cent. of Water.	Per Cent. of Ash.
Larva....	February...	3.06	0.250	91.72	7.06
	May.......	3.30	0.264	92.12	7.96
	June.......	3.15	0.311	89.13	7.33
	September..	2.57	0.182	92.93	9.53
	October....	2.83	0.264	90.66	8.62
	November..	3.20	0.285	91.03	8.10
Pupa.....	June.......	3.52	0.574	83.71	5.80
Adult.....	June.......	0.75	0.427	43.32	5.89

The adults yielded a much smaller live weight than either the larvæ or the pupæ because they possessed a much smaller proportion of water. Their dry weight was greater than that of the larvæ but smaller than that of the pupæ. The adults used for this weight were obtained from a large swarm on June 29, 1918, when pupation was very active, but there was no means of ascertaining their age; their weight probably decreases somewhat with age, and they live for a period of three to five days.

The ash of the larvæ varied from a minimum of about 7 per

cent. of the dry weight to a maximum of 9.5 per cent., the former being noted in February and the latter in September. The pupæ and adults yielded substantially the same percentages of ash, but these percentages were much smaller than in the larvæ.

There is more or less overlapping of the summer broods, which makes it difficult to estimate the number of larvæ produced during this season, but the winter crop of larvæ may be estimated with some degree of accuracy. In this investigation twenty-two samples of mud were obtained at the five regular stations in deep water during the month of November and they yielded an average of 17,350 *Corethra* larvæ per square meter of bottom. Five samples were secured in December also, and they gave an average of 21,900 individuals per square meter; but four of these samples were taken at Station II. which usually gave a larger yield than the other four stations. According to these figures, the early winter population of *Corethra* larvæ within the 20 meter contour may be conservatively estimated at 18,000 individuals per square meter. Between October and May the live weight averaged 3.1 milligrams per larva and the dry weight 0.266 milligram. Applying these weights to the above population gives a live weight of 55.8 grams per square meter, or 558 kilograms per hectare, which is equivalent to 497 pounds per acre, and a dry weight of 4.8 grams per square meter, or 48 kilograms per hectare, equivalent to 42.7 pounds per acre.

Muttkowski states that there may be two generations of summer larvæ in addition to the winter generation; but, since the former average somewhat smaller in size than the latter, the total weight of the summer broods is probably not greatly in excess of that of the winter brood. That is, a live weight of 1,200 kilograms per hectare (1,070 pounds per acre) would be a conservative estimate for the total annual production of *Corethra* larvæ in the deeper part of Lake Mendota; on this basis the dry weight would amount to somewhat more than 100 kilograms per hectare, or approximately 90 pounds per acre. These figures apply only to that portion of the lake which lies within the 20 meter contour line, since the larvæ are found in very much smaller numbers in the shallower water.

The 20 meter contour encloses an area of 664 hectares which would give an annual crop of larvæ amounting to substantially 797 metric tons, live weight, for this portion of the lake, or a dry weight of about 67 metric tons. The live weight of all other macroscopic inhabitants of this area was 92.3 metric tons and their dry weight was about 19 metric tons.

As previously indicated, the population of *Corethra* larvæ in the region between 8 meters and 20 meters averaged about one third as large per unit area as that in the deeper water. In order not to overestimate the annual production of the shallower water, the area lying between the shoreline and a depth of 10 meters may be omitted from the calculation since the number of larvæ found in this region is small; in addition, also, the average between the 10 meter and 20 meter contours, comprising an area of 1,738 hectares, may be reckoned as one quarter instead of one third as large as that of the deep water. On this basis the live weight becomes 300 kilograms per hectare and the dry weight 25 kilograms, thus making the annual crop of *Corethra* larvæ in this portion of the lake a little more than 521 metric tons, live weight, or about 43 metric tons of dry material. These results combined with those obtained for the deep water area give a total annual production of 1,318 metric tons of living larvæ which would yield 110 metric tons of dry material.

Chemical Results.

The results of the chemical analysis of the larvæ are shown in Table V. and they are stated in percentages of the dry weight.

Table V.

Results of the Chemical Analysis of the Larvæ of Corethra punctipennis Stated in Percentages of the Dry Weight.

Nitrogen.	Crude Protein (N×6.25.)	Ether Extract (Fat).	Crude Fiber (Chitin)	Per Cent. Ash,
10.74	67.12	9.45	6.15	7.96

The percentage of nitrogen is notably high, which means a correspondingly large proportion of crude protein. The percentage

given in the table does not include the nitrogen in the crude fiber (chitin) which amounted to 0.46 per cent. In comparison with this the larvæ of *Chironomus tentans* yielded a much smaller percentage of nitrogen, namely, 7.36 per cent.

The larvæ yielded a fairly large amount of fat (ether extract), namely 9.45 per cent. of the dry sample. Together the crude protein and the fat constituted more than 76 per cent. of the dry material. From the standpoint of quality, this large proportion of these two excellent food materials gives the larva of *Corethra punctipennis* a very high rank as a source of food for other organisms.

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BIOLOGICAL BULLETIN

MORPHOLOGY AND ORIENTATION OF THE OTOCYSTS OF GONIONEMUS.[1]

L. J. THOMAS.

Introduction.

Gonionemus is used as an example of the *Hydro-medusæ* in many zoölogical laboratories of this country. In spite of this fact relatively little is known regarding the morphology and orientation of the sense organs of the representatives of this genus. Both the location and the structure of the otocysts have been very imperfectly treated in the literature. Frequently morphological and experimental treatises mentioning the otocysts refer the reader to the works upon other genera, the otocysts of which are presumed to be essentially similar to those of *Gonionemus.* In studying specimens of *Gonionemus vertens* Ag. and of *G. murbachii* Mayer the writer was impressed by the lack of agreement between his direct observations and the published statements by various early workers. These discrepancies led the writer to investigate the problem further in an attempt to discover the source of the statements which have been so generally incorporated into the literature and to determine, if possible, the precise structure and relationships of the otocysts in this genus.

On the Pacific coast of this country *G. vertens* A. Ag. occurs in the Puget Sound region and on the Atlantic coast *G. murbachii* Mayer is found in abundance in the Eel Pond at Woods Hole, Mass. Many references in the older literature incorrectly refer to the Atlantic species as *G. vertens* because at that time the Atlantic form was not considered as specifically distinct from the

[1] Contributions from the Zoölogical Laboratory of the University of Illinois, No. 181.

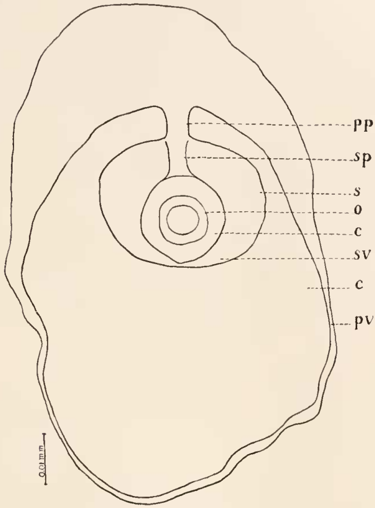
type of the genus. In 1895 Murbach mentioned certain differences between the two forms and in consideration for his work upon the Atlantic species A. G. Mayer (1901) described it as a distinct species under the name of *Gonionemus murbachii.*

The terms otocyst, statocyst, and lithocyst have been used interchangeably throughout the literature on Cœlenterates. In this paper the term otocyst is used in referring to the entire structure which is supposed to function as an organ of equilibrium. A careful study of the otocysts in *Gonionemus* has revealed the fact that the terminology ordinarily employed in describing the otocyst is entirely inadequate for an intelligible description of the parts and understanding of their functional relations. In *Gonionemus* the otocyst is not a simple vesicle enclosing an otolith as has usually been considered the case. From a morphological point of view there is considerable evidence that in *Gonionemus* the large vesicular structure is merely an adaptation for the protection of the true sensory apparatus all of which is lodged within the structure that has ordinarily been termed the otolith. This obvious confusion of terms renders a detailed description of the organ necessary.

Details of the organization of the otocyst are shown in Text Figure 1. The wall of the primary vesicle (*pv*) is the outermost wall of the entire organ and encloses a fluid-filled cavity (*c*) within which the spheroid (*s*) is suspended by the primary pedicel (*pp*). The thick wall of the spheroid encloses the fluid-filled cavity which is designated as the secondary vesicle (*sv*). Within the secondary vesicle rests the otolith (*o*), free to move about within the fluid-filled chamber. Extending from the distal end of the primary pedicel to the membranous lining of the secondary vesicle is a distinctly differentiated region to which the term secondary pedicel (*sp*) has been applied.

This investigation was carried on under the general direction of Dr. H. J. Van Cleave, to whom the writer is greatly indebted for suggestions and for securing the material and the identification of the species under consideration. Individuals of *Gonionemus murbachii* were obtained through the Supply Department of the Woods Hole Marine Biological Station for comparison

with those of *G. vertens* upon which the greater part of the work was done. Specimens of *G. vertens* collected at Friday Harbor, Washington, were submitted to Dr. Alfred G. Mayer and to Dr.



TEXT FIGURE 1. General organization of the otocyst of *Gonionemus*: *p v*, primary vesicle; *c*, fluid filled cavity; *s*, spheroid; *p p*, primary pedicel; *s v*, secondary vesicle; *o*, otolith; *s p*, secondary pedicel.

Henry B. Bigelow, both of whom very kindly verified the tentative identification of the species.

While the present paper deals primarily with the finer structure of the otocyst the latter part is devoted to a discussion of the orientation of the otocyst in the organism.

METHODS OF STUDY.

Specimens preserved in formalin were too opaque for accurate observations of the otocysts. Serial sections and toto-mounts of the bell margin, including ring canal, otocysts, and tentacles were prepared for the detailed study of the morphology and the orientation of the otocysts. The toto-mounts were made by

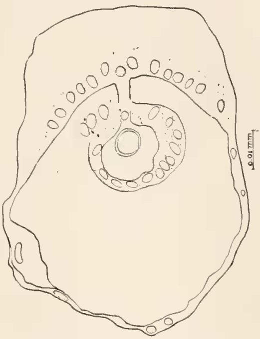
trimming the tentacles close to the bell margin with fine scissors and then clipping this rim, with the velum included, free from the remainder of the bell. Such preparations were stained with borax carmine and mounted in damar. The otocysts, though embedded in the mesoglea, were by this method sharply defined and readily visible for accurate observations. In some specimens prepared in this manner the finer details of structure could be determined. By careful manipulation, mounts prepared in this manner showed practically no distortion. Individuals 9 to 15 mm. in diameter were dehydrated, cleared, and mounted in damar with a shrinkage of 1 mm. or less in diameter. This shrinkage was obviously sustained uniformly by the various tissues for no evidences of wrinkling or distortion of parts were observable in the finished preparations. All drawings were made with the camera lucida from prepared mounts and sections.

Structure of the Otocyst.

Murbach (1903: 205) dismisses the anatomy of the otocysts in *Gonionemus* with the footnote: "The finer anatomy is naturally omitted. The description of the nervous system and the otocyst given in 'Das Nervensystem und die Sinnesorgane der Medusen,' O. u. R. Hertwig, text, pp. 48–69, Plates 4 and 5, fits very nearly those of *Gonionemus*." A careful examination of the Hertwig descriptions and drawings of otocysts in other genera reveals many points where a closer "fit" might be desired if the descriptions and drawings are to cover the conditions found in *Gonionemus*.

Superficial examination under a compound microscope reveals the otocysts of *Gonionemus* as tiny bubbles, each of which usually encloses a single spherical object. This last mentioned body has usually been considered as the otolith, though it probably comprises the entire sensory mechanism of the organ as indicated in the introduction to this paper. Detailed structure of this body is shown in Fig. 2, Plate I., which was drawn from a 15 μ section. This figure shows only the spheroid pendant from the wall of the primary vesicle by the minute primary pedicel (*pp*). The heavily nucleated cells which comprise the wall of the secondary

vesicle (*sv*) completely envelope the secondary pedicel (*sp*). The true otolith (*o*) is shown within the secondary vesicle. In the section from which this drawing was made a small tangential slice such as is shown in Fig. 3, Plate I., had been cut from the surface of the spheriod thus disclosing the cavity of the secondary vesicle. No sensory hairs or ridges were demonstrated. Perkins (1902: 786) also calls attention to the absence of such structures, though his observations were probably confined to the walls of the primary vesicle. In sections a thin tangential slice from the surface of the spheroid, Text Figure 2, may at



TEXT FIGURE 2. Tangential slice only partly removed from wall of spheroid.

first glance remind one of the projections figured by Hertwig (1879: 183, Figs. 1 and 2) for *Carmarina*.

The primary pedicel measures 0.043 mm. in diameter and 0.069 mm. in length. At its proximal end a single large nucleus is found. This pedicel is very delicate for in sectioning, it, together with the spheroid, is often torn loose from the cyst wall. In other cases only the spheroid is torn away from the distal end of the pedicel and may be found elsewhere on the slide. Goto (1903: 8) records encountering the same difficulty in the preparation of sections.

The secondary pedicel measures 0.115 mm. in length. Its diameter near the middle is about 0.023 mm. but the distal end is

expanded to about 0.043 mm. Two nuclei of apparently fixed relationships are found in the secondary pedicel, one near the proximal and the other near the distal end.

The cavity of the secondary vesicle is normally spherical in form though it is capable of some distortion. The diameter is about 0.138 mm. The contents of this cavity take stains so lightly and so evenly that there is strong evidence that only a fluid is present. Two or three concentric rings are observable in the spherical otolith contained within the secondary vesicle (Figs. 4–5, Plate I.). There is considerable variability in the size of the otolith. Some of the largest measured 0.069 mm. in diameter. The fluid in which the specimens were preserved was distinctly acid, consequently any calcareous deposits that might have been present in the otolith had been destroyed, leaving only the supporting structures. Perkins (1902: 786) refers to the otolith as "a calcium salt deposit in an organic matrix." In the specimens examined the otolith had no fixed position within the secondary vesicle but was apparently free to move about in the fluid filled space. The entire sensory mechanism as ordinarily described for an otocyst is thus contained within the confines of the spheroid. In the light of this morphological evidence it might be easily possible that the destruction of the primary vesicle need not appreciably impair the functioning of the organ.

Murbach (1903: 206) in experiments upon the function of the otocysts of *Gonionemus* collapsed the "otocysts" by thrusting them with a fine needle. In all probability the injury inflicted did not extend beyond the collapsing of the primary vesicle. According to his statements the specimens continued to act normally after this treatment. Upon the results of these experiments and upon the behavior of a single individual from which the otocysts were excised he based his conclusion that the otocysts play no important part in establishing the equilibrium in *Gonionemus*. The more or less normal behavior of the much mutilated individual from which the otocysts were cut is not readily explainable. On the other hand, in view of the fact that morphologically the entire sensory mechanism of the otocyst seems to be confined to the secondary vesicle there is little reason to expect serious

interference with the functioning of that organ when only the protective primary vesicle is collapsed.

My observations upon specimens of *G. vertens* and of *G. murbachii* have failed to disclose any essential difference in the details of structure or in the general orientation of the otocysts of these two species.

Relations of Otocysts to Bell Margin.

Because of the reliance placed upon the works of previous investigators and writers on the subject, Mayer's "Medusæ of the World" includes several erroneous statements and incorrect figures of the otocysts of *Gonionemus*. Few authors, with the exception of Mayer, have attempted to describe definitely the location of the otocysts with reference to their position on the margin of the bell. It seems probable that his description is based chiefly on Perkins' publication (1903: 786) which has been extensively quoted by Mayer though his observations upon the otocysts are very misleading. In Plate 34, Fig. 19, Perkins has reproduced a drawing by Professor Brooks which shows the otocysts as external projections from the margin of the bell between the bases of the tentacles. Further, the drawing of the "radial transverse section" of the bell (Fig. 25 of his same plate) confirms the impression of their external location. His explanation of their origin is as follows:

> In the case of the sensory clubs, the endodermal tissue of the circular canal grows down in a plug into the ectodermal tissue of the bell margin. This latter becomes closely applied to the outside of the plug, as a thin investing epithelium, and it also spreads out in a thin lamella over the inner surface of the capsule which appears in the ectoderm of the developing club.

In his summary Perkins (1903: 789) says, "sense organs appear at determinate points on the bell margin." The work in this article on sense organs seems to be principally that of Professor W. K. Brooks whose observations were apparently accepted by Perkins without attempt at verification.

Mayer (1910: 341) in his synopsis of the genus seems to have incorporated the foregoing incorrect observations bodily for he refers to the otocysts as "lithocysts external." On page 342 of the same work he again states that there are "numerous ex-

ternal lithocysts upon the bell margin between the tentacles." In characterizing the genus *Cubaia* (page 351), he states that there are "lithocysts projecting outward as in *Gonionemus*". . . "this genus is closely related to *Vallentinia* Brown 1902 but in *Vallentinia* the lithocysts are enclosed and on the inner side of the margin, whereas in *Cubaia* they are external and on the lower side of the margin between the tentacles." The plates of the same monograph are just as confusing as the foregoing descriptions in the manner in which the otocysts are located. Otocysts are shown as distinct projections from the external margin of the bell in Fig. 1 of Plate 45, though Fig. 2 of the same plate and Fig. 3 of Plate 46 correctly portray them embedded in the mesoglea as outgrowths from the ring canal.

The location of the otocyst within the mesoglea and the orientation with reference to other structures is shown in Fig. 1, Plate I.

Relations of Otocysts to Tentacles.

Hargitt (1910: 249) referring to the location of the otocysts, says: "Normally they should occur in somewhat symmetrical order between the bases of the tentacles. This, however, is rarely the case." Mayer (1910: 342) gives the number of tentacles for specimens of *G. vertens* 15 mm. in diameter as 60 to 70 but it appears that his count is too low. In the following table are given the data regarding the numbers of otocysts and tentacles in part of the material studied in the present investigation.

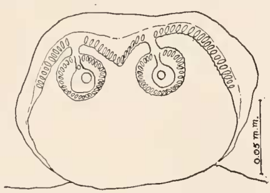
Table I.

Relative Numbers of Otocysts and Tentacles in Gonionemus.

Specimen Number.	Diameter of Bell After Clearing.	No. of Tentacles.	No. of Otocysts.
G. vertens			
1	14.5 mm.	91	76
2	14	86	73
3	14	99	72
4	12	84	60
5	11	94	75
6	11	101	78
7	10	94	89
8	9	79	61
9	9	83	57
10	8	79	60
G. murbachii			
1	9	48	61
2	10	64	70

With reference to the tentacles these organs display no absolutely fixed relationship. Though they usually alternate with the tentacles they may occur in pairs between two adjacent tentacles or two or more tentacles in continuous sequence may have no intervening otocyst.

In *G. vertens,* nos. 6 and 7 had three paired otocysts between adjacent tentacles; no. 7 also had two abnormal otocysts with two



Text Figure 3. Abnormal otocyst with two sensory spheroids within the same primary vesicle.

sensory spheroids in each capsule Text Figure 3. Hargitt (1901 : 249) has noted this last mentioned variation and has called attention to its relative infrequence. Specimen no. 1 of *G. murbachii* had thirteen paired otocysts and no. 2 of the same species had eight.

Summary.

1. There are two distinct vesicles in the otocyst of *Gonionemus.*
2. It seems probable that the conspicuous primary vesicle has chiefly a protective function, enclosing the essential sensory mechanism.
3. The secondary vesicle contains the otolith within a fluid-filled cavity.
4. No sensory hairs or ridges have been demonstrated.
5. Puncture of the primary vesicle as practiced by Murbach and others probably does not impair the essential sensory mechanism.
6. The otocysts of *G. vertens* and of *G. murbachii* are in close proximity to the periphery of the ring canal, their capsules imbedded in the mesoglea so that no portion of them protrudes beyond the margin of the bell.
7. The number and arrangement of otocysts and tentacles in *Gonionemus* is variable.

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EXPLANATION OF PLATE.

SYMBOLS.

c, cavity of secondary vesicle,
e c, ectoderm,
e n, endoderm,
e x, exumbrella,
m, mesoglea,
n, nerve ring,
n c, nettling cells,
o, otolith,
p p, primary pedicel,
r, ring canal,
s, spheroid,
s l, supporting layer,
s p, secondary pedicel,
s u, subumbrella,
s v, secondary vesicle,
v, velum.

PLATE I.

The otocyst of *Gonionemus vertens*. All drawings were made with the camera lucida. The projected scale indicating magnification in each instance has the value of 0.01 mm.

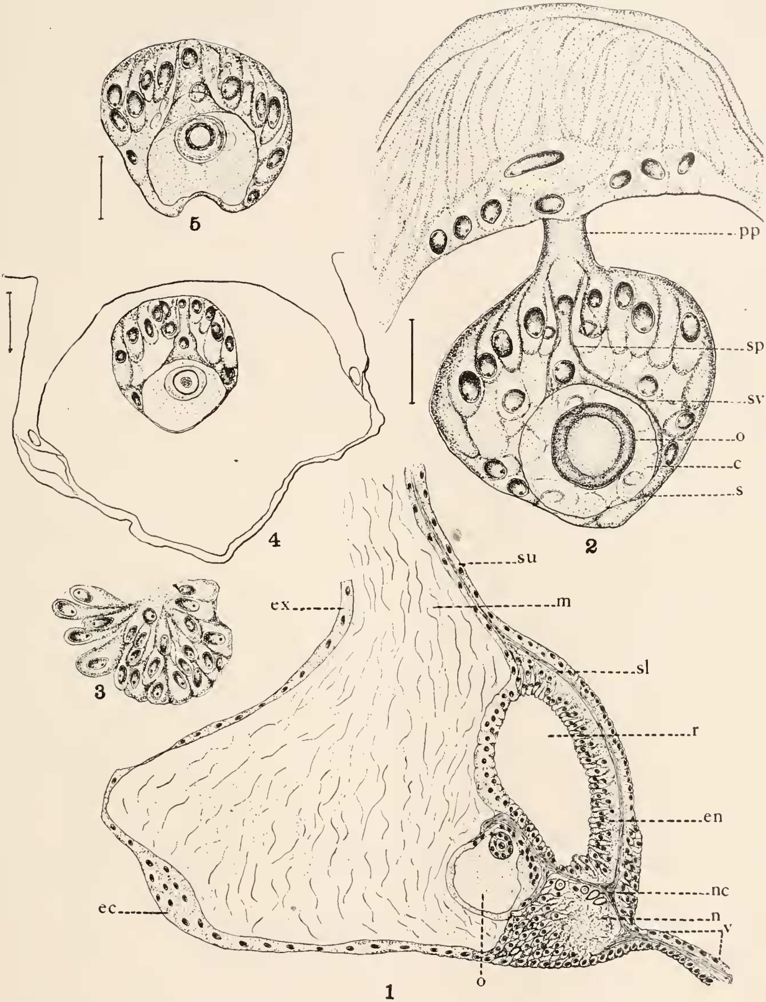
FIG. 1. Section through bell margin showing general location of the otocyst within the mesoglea and its orientation with reference to ring canal.

FIG. 2. Longitudinal section through the spheroid of an otocyst showing histological details.

FIG. 3. Tangential slice from the wall of the spheroid similar to the section removed from the front surface of the wall of the secondary vesicle in the foregoing figure.

FIG. 4. Longitudinal section through an otocyst to show the concentric rings within the otolith.

FIG. 5. A spheroid with a portion of the wall of the secondary vesicle removed.



L. J. THOMAS.

A SYMBIOTIC FUNGUS OCCURRING IN THE FAT-BODY OF *PULVINARIA INNUMERABILIS* RATH.[1]

CHARLES T. BRUES AND RUDOLF W. GLASER.

During the late winter and spring of 1920 the present writers became interested in the supposedly symbiotic organisms which occur in various scale insects, hoping that they might be able to propagate some of them in artificial cultures and learn something of their physiological activities. From knowledge gained thus, it seemed probable that they might be able better to determine whether such organisms exist in the insects as true symbionts, as mere commensals or as innocuous parasites.

As is well known through the investigations of several workers, the entrance of the symbionts[2] into the egg of scale insects can be readily followed as well as their behavior during embryological development. A good account of this has been given by Shinji ('19) who also includes a summary of the previous work of other authors.

In the nymphal or full-grown scales of many species it is more difficult to find and interpret the symbionts and it seems probable that in some cases they must either become very much reduced in numbers, very highly modified, or perhaps reduced to minute and unrecognizable spores or granules.

Several workers who have recently examined the symbionts of Coccids (*e.g.*, Buchner '12, Sulk '10, Teodoro '18) regard them as yeasts (Saccharomycetes), although Berlese ('06) referred one species to the genus *Oöspora,* one of the *Hyphomycetes.*

Before beginning our work with *Pulvinaria innumerabilis,* the cottony maple scale, we examined several other species of Coccids, but were unable successfully to cultivate the symbionts from these, with the possible exception of one of the pine scales,

[1] Contribution from the Entomological Laboratory of the Bussey Institution, Harvard University, No. 176.

[2] We have used this name as a convenient designation already in current use and will discuss its appropriateness on a later page.

Chionaspis pinifoliæ Fitch. From the latter we isolated an organism, perhaps related to *Oöspora,* but we could not secure it with sufficient regularity to satisfy ourselves that it was really the symbiont, and not a contaminating species of microörganism of which one always encounters numerous species in work of this kind. Just how closely related the symbionts of various Coccids may be, must remain a matter of doubt until a considerable number have been carefully investigated, and preferably cultivated also, but our own observations lead us to think that more than one type of organism will be found after careful, systematic study.

In the following brief review of literature we have considered only such contributions as appear to bear directly on symbionts quite probably closely related to the form with which we have worked.

The first reference to the occurrence of symbiotic organisms in Coccidæ is that of Leydig ('54) who found discrete, lanceolate bodies which he believed to lie free in the lymph of *Coccus* (now *Lecanium*) *hesperidum.* He described them as 4 μ in length, multiplying by buds which do not separate till they have attained the size of the parent cell. Neither at this time, nor in 1860 in his contribution to the development of the Daphnids, did he realize their significance. From the rather large size and method of multiplication, these are probably similar to the organisms found in *Pulvinaria,* which also appear in freshly mounted smears as though they were free in body fluid. Putnam ('79) studied in considerable detail the biology, anatomy and development of *Pulvinaria innumerabilis* in Iowa. In connection with a section devoted to the contents of the ovaries he gave an account of the organisms with which we deal in the present paper. His observations were so carefully made that we have thought it worth while to include the following resumé. On opening a female at any time from October to May, five classes of bodies are set free, all of them apparently associated with the development of the eggs. These are: First, a clear protoplasmic liquid; second, clear spherical globules 10 μ–30 μ in diameter, lighter than water, and not taking the ordinary aniline stains. He was undoubtedly cor-

rect in believing these to be yolk and fat globules. Third, exceedingly minute, apparently spherical bodies, heavier than water and staining with eosine. Putnam thought that these might be bacteria although he suggests that they may be comparable to the blood-disks [erythrocytes] of vertebrates or that they may be stages in the development of the fourth class of bodies which he next considers. That all of these suppositions are probably incorrect will appear from our account on a later page. Fourth, small oval bodies $3\,\mu$–$5\,\mu$ in diameter and $10\,\mu$ long and heavier than water. These represent the organism which we have studied but Putnam was unable to decide whether they were spermatophores or whether they corresponded to the pseudonavicellæ observed by Leydig in *Lecanium,* as had been suggested to him by Dr. E. L. Mark.[1] A fifth class of bodies observed were the small incompletely formed eggs.

There can be no doubt that Putnam found the fungus with which we have worked as his description and figures make this point very clear. As he found it in all cases, it is further clear that the symbiont enjoys a wide range since his observations were made on specimens collected in the middle west and our own on material from eastern Massachusetts.

Metschnikoff ('84) found in a crustacean, *Daphnia magna,* a fungus which he called *Monospora bicuspidata.* This he regarded as a parasite, but recent developments in the study of apparently similar organisms in insects, suggests that these Crustaceans should be reëxamined.

Moniez ('87) described a fungus which he called *Lecaniascus polymorphus,* occurring in the scale insect, *Lecanium hesperidum.* He refers to Leydig's 1854 paper, mentioning the fact that *Lecaniascus* is evidently the same as Leydig's pseudonavicellæ. Moniez speaks of the organism as a parasite, and he found it in all specimens, both young and old, of the *Lecanium* that he examined. He described the isolated cells as 4–$5\,\mu$ in length, and found mycelia attaining a length of 50–$60\,\mu$. Some doubt is cast upon this author's conclusions by Vejdovsky ('07) who suggests that Moniez may have seen two microörganisms, one represented

[1] Mark ('77) does not consider these bodies, however, in his paper on the anatomy and histology of the Coccidæ.

by the single cells and another by the mycelia and asci, the latter perhaps *Alternaria tenuis,* a parasitic fungus that attacks various Coccids of the genus *Lecanium*.

Lindner (1895) found in an European scale insect (*Aspidiotus nerii*) a yeast-like organism which he regarded as related to *Saccharomyces apiculatus*. By crushing one of the insects between a slide and cover-glass he observed large numbers of the yeasts, both between the small masses of fat-body and actually in the adipocytes. The organisms he described and figured as usually very long, pointed at one end, or lanceolate, sometimes joined in pairs by their acute tips, and frequently budding after the manner of yeast cells. At that time Lindner was unable to cultivate the organism although he evidently regarded it as a parasite, naming it *Saccharomyces apiculatus,* var. *parasitus*. He found it forming a mass at the posterior pole of the egg and made an ingenious explanation for its presence there, suggesting that one of the pointed tips perforated the egg and gave off a bud which then multiplied to form the mass or mycetome.

A later paper by Lindner ('07) which appeared in the *Wochenschrift für Brauerei* we have not seen, but from published reviews of this, it appears that it contains nothing bearing on the physiology or systematic position of his yeast-like organism.

Berlese ('06) studied in detail an organism which he found in *Ceroplastes rusci,* and was successful in growing it on artificial media. From Berlese's account, it appears that the fungus, which he calls *Oöspora saccardiana* is very similar to the one described by us in the present paper. The yeast-like cells in the Coccid vary in length from 4–12 μ, sometimes attaining a length of 16–18 μ in early summer, agreeing in size and also in form with those we have observed in *Pulvinaria*. In culture there is a great similarity in the general morphology and development of mycelia, and although Berlese gives few details concerning cultural characteristics, at least one statement shows a striking difference between the two. He found that although the symbiont from *Ceroplastes* grows rapidly on gelatine media, that these are not liquified, while as will appear from our account, the *Pulvinaria* fungus exhibits a powerful liquefactive action on gelatine.

Concerning the distribution of the symbionts in the body of *Ceroplastes,* Berlese gives no account, except to state that they generally occupy the visceral cavity completely in all individuals, in numbers estimated at from 60,000–70,000 cells.

Two other genera of soft scales, *Kermes* and *Physokermes,* have been the subject of investigation by Sulc ('07) and Vejdovsky ('07). The former found two distinct symbiotic organisms in two species of these Coccids, readily distinguishable from one another on the basis of size and form. These he described as representatives of a new genus, *Kerminicola.* Vejdovsky regarded them as Saccharomycetes in which opinion Sulc concurred. The microscopical structure of the symbionts was carefully described and is similar to that of the species in *Pulvinaria* studied by us, although *Kerminicola* evidently has a much more prominent and discrete nucleus and fewer multinucleate cells; also the form of the cells is generally much more elongate. Vejdovsky found the symbionts in the fat cells of the host in large numbers and observed them freed in the hæmolymph as a result of a disintegration of the adipocytes which he believed due to the activity of the included organisms. He, therefore, regarded them as parasites, but pointed out that their activities do not affect the gonads of the host, nor the development of the eggs in its body. They do, however, serve to break down the fat and to consume the remaining tissues of the host's body, after which it remains a shell for the protection of the now fully developed nymphal *Kermes.*

In a later paper, Sulc ('10) gives a more extended account of the similar organisms of a number of Homoptera and speculates at considerable length upon the relations between the insects and fungi. After more extended study, his ideas have been considerably modified and he has come to regard the microörganisms as essentially symbiotic in their association with their host insects. He suggests that the production of enzymes by yeasts (for he still refers the symbionts to the Saccharomycetes) must be considered in any interpretation of their physiological relations to the insects. Apparently he made no attempts at cultivation *in vitro.*

Pierantoni has considered the symbionts of certain Coccidæ in

several papers of which that of 1910 in the *Zoologischer Anzeiger* is of greatest interest in the present connection. In *Icerya purchasi,* he traced the entrance of the organisms into the egg of the scale insect and its subsequent behavior through the formation of the polar mass in the egg to the development of the mycetocyte in the larva. He found that the individual symbionts of this species were at first round or oval, and not noticeably elongated, and that later during embryonic development and at the time of hatching they became quite inconstant in form, varying from rounded or oval to much elongated and frequently strongly curved cells, all, however, of about the same diameter. These long forms may fragment, each piece becoming a new individual, while the short ones commonly divide by fission into equal parts. Occasionally, however, Pierantoni found cells multiplying by budding in the body cavity of the host, and more rarely in the mycetome.

On a gelatine medium, with high sugar content (20 per cent. saccharose) he was able to cultivate a yeast-like organism from the mycetocytes. These developed after four days' incubation as colonies that are described as small spheres in the gelatine which develop a sort of finger-like process which becomes prolonged toward the surface of the gelatine and then emerges projecting above it in the form of a finger, or with the base enlarged and pyriform. The individual organisms were of yeast-like form with buds more or less developed. It thus appears that the organism obtained in culture by Pierantoni differed greatly in form, size, and method of multiplication from the organisms in the insects. as will be shown later, the cultures obtained by us from *Pulvinaria innumerabilis* exhibit no such remarkable distinctions, in morphology and reproduction, from the cells in the host insect. Although he makes no mention of the development of mycelia in his cultures, the form of the colonies indicates without question that such must have been present, and that if the organisms in the mycetome were actually those obtained in culture, the symbiont of *Icerya* is a true fungus.

The Symbionts of the Half-grown Pulvinaria.

In early April the overwintered cottony maple scale-insects are partly grown and may be found attached to the bark of small twigs on the food plants, which consist of various maples and a few other woody plants. At this time they are nearing the end of their period of hibernation and do not yet exhibit any active growth.

If a specimen in this condition be crushed on a slide in normal salt solution, the symbionts may be readily seen free in the liquid. They are heavier than the medium and fall next to the slide, thus separating from the released fat globules which accumulate above, against the cover-slip. In such a preparation all the organisms appear to be in the liquid, as those in the fat cells are not readily discernible on account of their hyaline nature. This, no doubt, accounts for the statements that the symbionts occur in the lymph rather than in the tissue.

In sections it is evident, however, that the organisms are absent, or at least very nearly so, from the blood and that they are very generally distributed through the fat body, imbedded in the adipose cells. They are usually spaced in a quite regular way showing that they migrate or at least change their position in the cells subsequent to multiplication. The density of distribution is well indicated in the drawing (Fig. 1, *A*) which is made from a section of $6\,\mu$ in thickness where all of the symbionts present have been sketched. The photograph on Plate I., Fig. 1, is made from a typical cross-section through an entire insect, in which the symbionts appear as minute oval dots. On Plate I., Figs. 2, 3 and 4, are reproduced several small areas of the fat-body viewed at higher magnification with their symbiont inclusions. Sketches of a few still more highly magnified symbionts are shown in Fig. 1, *B*. Here it will be seen that they are extremely variable in size and shape, but always quite distinctly oval in form with one pole more acute and the opposite one more rounded. They vary in length from 10–16.7 μ by 5–6.5 μ in width, with an average size of 10–12.5 μ by 5.7 μ. Budding forms are frequently present, the bud developing at the narrow pole and separating either as a small oval, or more rarely rounded, cell. The buds at the time of sepa-

ration are of the same general shape as the cells from which they originate, but much smaller, varying in length from 6.2–6.7 μ. Some buds are nearly round, in which case they separate when considerably smaller, about 3.7 μ in diameter.

FIG. 1. *a*, Section of fat body showing symbionts *in situ* in overwintered nymphal *Pulvinaria*, $\times$ 260; *b*, isolated symbionts, stained with methylene blue and eosin, $\times$ 1,000.

The internal structure of the symbionts shows very little uniformity. Sections of tissue fixed in Zenker's solution and stained with methylene blue and eosin show one or several rather distinct deeply stained portions that resemble nuclei. When one is present it is usually central, when two or more are present, they are separated rather evenly from one another and from the cell wall. The remaining protoplasm shows irregular denser streaks and spots of irregular size, with usually one large, several smaller, or one large and one small vacuole, with generally a number of minute clear spots that can be seen only after very close examination. We have tried to bring out other details of structure by different methods of fixation and staining but without much success. Smears fixed by such methods as drying and submersion in absolute alcohol and subsequent staining with Giemsa's stain, Manson's Protozoan stain and carbol-fuchsin, give quite similar pictures to those in sections which are disappointing from a cytological standpoint. Evidently the symbionts are of very delicate consistency. No chains of symbionts occur at this time (early April) except for the single attached buds, and no masses of contiguous ones are to be found.

Changes in the Symbionts after Hibernation.

Our observations on these are very fragmentary as our available time at this season was occupied with the cultural experiments described below. It appears quite certain, however, that the oval symbionts of the early spring *Pulvinaria* nymphs undergo further multiplication and morphological changes in late April, and during May. A cursory examination of specimens about May 10 showed the symbionts in groups sometimes forming short strings, somewhat similar to the typical mycelium which was obtained in cultures. The picture at this time indicates quite clearly the fungous nature of the yeast-like organisms just described, found before spring growth begins.

Isolation of the Fungus from Pulvinaria.

Our first attempts to cultivate in artificial media the yeast-like cells present in *Pulvinaria* were made in early April. The overwintered, partly grown scales were brought into the laboratory still attached to the maple twigs on which they occur. The scales can readily be detached from the twig by means of a sterile needle and allowed to fall upon a sterile microscope slide. The scales thus removed, were treated in several ways as experience had taught us that material of this sort is very apt to be contaminated on the surface by various microörganisms. Some were treated with 85 per cent. alcohol for a few minutes, others rapidly passed through the flame of a Bunsen burner, and others were used without treatment, except to avoid contact with any unsterile object. Our first media were potato agar and "sugar agar" which consisted of potato agar to which varying amounts of maple sugar (2½ per cent.–20 per cent.) had been added. These tubes are readily inoculated by crushing the scale insect between two microscope slides and streaking the agar with a loop dipped in the body-juices thus extracted. It is also easy to drop the whole insects into culture tubes and then crush them on the agar by means of a dissecting needle. From a large series of tubes inoculated according to these methods, nearly one half showed after three days a good growth, white in color, spreading on the surface of the media. These colonies appeared to de-

velop almost equally well in the greatly diverse concentrations of sugar and in the plain potato agar. A microscopic examination at this time showed that nearly all the tubes in which growths occurred contained the same microörganism, and that only a few were contaminated by molds (*Penicillium*) and bacteria. The abundant species showed large numbers of budding yeast cells like those in the living scale insects and the development of mycelium as well showing that the symbiont was a fungus and not a yeast as one might otherwise be led to believe from a study of the living insects, at this season of the year when only single budding cells occur in the fat body.

Several of the colonies thus obtained were plated, found to be pure and sub-cultures were then made from these which have furnished the material for the description and cultural characters detailed below. Although the morphology of the organism in the living insects and in the cultures and the fact that it was recovered in such a large proportion of the cultures, left little doubt as to the identity of the two, we undertook some serological tests to corroborate if possible the conclusion based on morphological data.

For this purpose, two rabbits were secured, inoculated with bouillon cultures of the fungus, and serum from each was tested with the cultures and also with the organisms in the living scale insects. The following table shows the doses used and the reactions of the rabbits.

Date.	Amount of Culture.	Weight.	
		Rabbit A.	Rabbit B.
April 13	3 c.c.	2185 gms.	1905 gms.
April 16	5 c.c.	2180 gms.	1910 gms.
April 20	5 c.c.	2080 gms.	1910 gms.
April 24	6 c.c.	2110 gms.	1895 gms.
April 29	6 c.c.	2030 gms.	1915 gms.

One week later some blood was withdrawn from each rabbit and serum prepared. A precipitin test with the culture gave a positive reaction after four hours, and agglutination was very pronounced after one and one half hours as examined under the microscope. On account of the impossibility of securing sufficient material from the living scales, it was possible to try with

them only the agglutination test. With these the reaction was not so pronounced as with the yeast-like forms in culture, but nevertheless distinctly positive. Unfortunately by the time the animals had been immunized (early May) the number of yeast-like cells in the insects had decreased and the reaction could not be so readily observed as in the cultures, or so well as it might have been several weeks earlier in the spring when the insects contained innumerable, separated, oval cells. The data from the rabbit experiments has, however, convinced us that there can be practically no question that the organism cultivated is actually the one present in the insects. Furthermore, since we have never failed to observe it in living scales from this locality, and since Putnam (v. *antea*, p. 301) found it invariably present in Iowa, it is undoubtedly present regularly in *Pulvinaria innumerabilis*.

Cultural Characteristics of the Fungus.

As stated at the outset, we wished to grow the symbiont on artificial media, not only to describe it adequately, but to determine as completely as possible its physiological activities. In order to do this, sub-cultures from the original isolations were planted upon various media, such as are in general use by bacteriologists and mycologists. From these, the following observations were made.

Growth on Solid Media.

Potato Gelatine Colonies.—Growth rapid; after 72 hours, cottony with flocculent elevated center and filamentous edge, diameter of center 1 mm., width of margin 1 mm. Liquefaction cup-shaped.

Nutrient Gelatine Colonies.—Growth slow; after 72 hours, rounded, with central elevation. Diameter 0.3 mm., with roundly lacerated edge. Liquefaction cup-shaped.

Potato Agar Colonies.—Growth rapid; after 72 hours, filamentous, ciliate (sub-surface) or rounded (surface). Disk when present, smooth; elevation convex; edge of round colonies smooth, that of irregular colonies radiately filamentous or ciliate. Internal structure finely granular. Diameter 1.5–2.5 mm.

Nutrient Agar Colonies.—Growth slow; after 72 hours, round,

with smooth surface and convex elevation. Edge smooth; internal structure finely granular, with irregular central core. Diameter 0.6 mm.

Potato Gelatine Stab.—Liquefaction begins in 48 hours; growth best at tip, the line of puncture filiform; liquefaction at first napiform, becoming stratiform, surface umbonate. Medium unchanged.

Nutrient Gelatine Stab.—Same, but liquefaction proceeds more slowly and is more nearly crateriform.

Potato Agar Slant.—After 72 hours growth is abundant, spreading, densely rhizoid, convex. Color white, opaque, surface glistening, smooth. No odor, consistency viscid. Medium unchanged.

Nutrient Agar Slant.—After 72 hours growth is moderate, beaded, with a few rhizoid colonies where medium is thin. Surface smooth, glistening. Color white, opaque. No odor, consistency butyrous. Medium unchanged.

Locke's Agar Slant.—Growth like other agar slants, scanty, beaded, many rhizoid colonies, consistency butyrous. After a much longer incubation (four weeks) there is not a very heavy growth, but it is still more or less beaded and highly rhizoid on the sides and extending deep into the agar.

Molisch's Agar Slant.—After 72 hours growth abundant, much like that on potato agar. Consistency very ropy. No pigment, even in old cultures.

Potato Agar (with Yeast[1]) Slant. Growth after 72 hours abundant, like that on potato agar, but with fewer rhizoids.

Potato (Pieces).—Colonies raised, slightly yellow, growth good. There is a trace of ammonia.

Growth in Liquid Media.

Nutrient Bouillon.—After 48 hours growth good; after 72 hours, no surface growth, no clouding and no odor, but with an abundant viscid sediment. No hydrogen sulphide is produced.

Locke's Solution.—After 48 hours not very much growth; after 72 hours, no surface growth, no clouding and no odor, but with an abundant viscid sediment. After a much longer incuba-

[1] Made by adding 2 per cent. of thoroughly crushed bread yeast.

tion at room temperature (four weeks), the growth becomes very flocculent and adheres to the surface of the glass where it is finely dotted with very dark, pigmented spots.

Molisch's Solution.—After 48 hours rather good growth; after 72 hours, no clouding or surface growth, but with very abundant, slightly viscid sediment, no odor.

Sugars.—We have grown the organism in six sugars: lactose, dextrose, mannite, saccharose, levulose and maltose. None of these, however, furnish any differentiating characters; in all there is good growth without the formation of gas, but with heavy viscid sedimentation, and after prolonged incubation, the development of a distinct surface film.

Milk.—Growth is abundant, and after four to six days incubation the milk begins to clear at the top, sediment collecting in the lower half of the liquid. Litmus milk becomes distinctly red at the time of clearing. After about a week, the liquid becomes whey, with a sediment at the bottom of the tube.

Anaërobic Media.—We have not been able to cultivate the organism under anaërobic conditions.

Production of Enzymes.

Protease.—Gelatine is rapidly liquefied. Milk cultures, tested after 20 days, give a positive reaction with $MgSO_4$, NaOH and $CuSO_4$, showing the presence of peptones.

Lipase.—After both 20 and 30 days' incubation, cultures in either whole or skimmed milk give positive reactions. There is a strong odor of butyric acid. Ethyl butyrate is also decomposed with the formation of butyric acid. The 30-day culture was tested with pyrogallol and stannic chloride and gave a positive reaction also.

Diastase.—Bouillon cultures after ten days' incubation were treated with starch paste at incubator temperature for 48 hours; after this, Fehling's solution was reduced, demonstrating the presence of sugar.

Morphology of the Cultivated Fungus.

The cultures show during the first few days only yeast-like budding cells like those seen in the early spring in the fat-body

of the insects. These vary considerably in size, ranging from 6–16 μ in length and from 3–9 μ in width. They are thus more variable in size in culture than in the insect and generally more elongate. The maximum length is about the same, but there are more smaller cells in culture, due no doubt to the fact that during rapid development the buds separate when less fully developed than in the insect. The internal structure when stained, appears to be the same as that of the forms in the fat-body described above.

After prolonged incubation on solid media the formation of a distinct mycelium always occurs. This is at first white, but after several weeks, blackened spots sometimes become visible, due to the development of pigment in the walls of certain groups of cells. This occurs especially on potato-agar. In at least one liquid medium, Locke's solution, the same blackened cells develop.

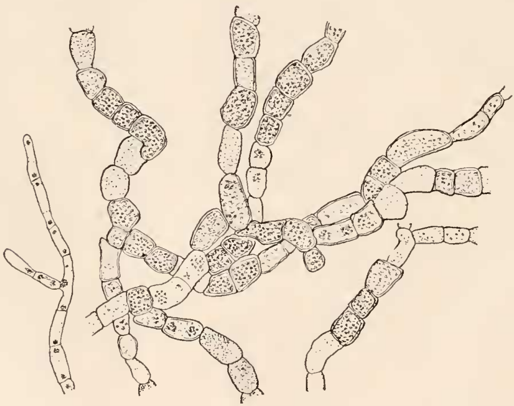


FIG. 2. Portion of mycelial growth of symbionts after prolonged incubation (10 days) in liquid bouillon medium. Magnified about 400 diameters.

The mycelium (Fig. 2) is branched and of quite irregular form. The larger hyphæ measure from 6–15 μ in diameter, broad and narrow cells frequently alternating or with one size interpolated in series of the other. Some cells, usually single ones or pairs, more rarely several in succession are heavily pig-

mented and appear very dark in fresh material; a pair of these are frequently rather closely fused to form an oval body. The contents of these dark cells are highly granular, with the protoplasmic mass clearly separated from the cell wall by a hyaline layer. Toward the tips the hyphæ are usually much more slender, pale in color and with only scattered granules in the protoplasm. The method of branching is entirely dichotomous, many lateral branches along the larger hyphæ consisting of but a single cell although both near the tips and on the larger hyphæ there are many long branches which again subdivide. Very rarely there is an anastomosis of the finer apical branches formed by lateral prolongations of the cells. In preparations we have had difficulty in recognizing the conidiophores, but free conidia are present in old cultures. They measure from 8–10 μ in length and are broadly oval in form, very nearly transparent and without color.

Systematic Position of the Cultivated Fungus.

We have been unable to deal with this matter in a satisfactory way owing to our unfamiliarity with cryptogamic botany and must leave it for consideration by an experienced mycologist. It is very evident that the symbiotic organism in *Pulvinaria* cannot be regarded as a Saccharomycete, although its morphology and method of multiplication in the insect does not preclude such an assumption. Indeed, the symbionts that have been observed in other Coccids and in most other Homoptera as well have usually been regarded as yeast-like organisms and commonly referred to the genus Saccharomyces or to new genera located in the same group of plants. Berlese ('06) has placed the organism which he cultivated from *Ceroplastes* in the genus *Oöspora,* thus recognizing it as a true fungus, but hitherto, with the possible exception of Pierantoni ('10) no one else seems to have been successful in growing *in vitro* any of the symbionts of coccids.

The species which we have obtained from *Pulvinaria* seems to be quite similar to the one described and figured by Berlese from *Ceroplastes* so far as the general morphology of the yeast-like cells in the coccid and the mycelial structure in culture. Neither species, however, has been sufficiently studied to make a more posi-

tive statement. Dr. O. F. Burger kindly examined some of our cultures and has expressed the opinion that they probably represent a species of *Dematium* or a related genus. Such morphological characters as we have been able to make out agree well with descriptions of this genus to which it may be tentatively referred.

Physiological Rôle of the Symbiont.

As stated at the outset, we have attempted to determine the physiological behavior of the *Pulvinaria* symbiont in culture to ascertain in what way it may affect the metabolism of the coccid.

Contrary to what occurs in the case of most yeasts, this organism produced no gas in media made from any of the sugars in which it was grown. This is quite what might be expected as the coccid tissues are undoubtedly rich in sugars and any organism producing gas in the presence of such substances could not be tolerated in the body of the coccid.

On the other hand a diastatic ferment is produced in quite appreciable quantities. Whether this bears any relation to the metabolism of the coccid is not entirely clear. In the adipose tissue and body liquids, starch is probably not present to any considerable extent, although in the large quantities of plant sap ingested by the coccids there must be substances upon which this ferment might act. It has been shown also by Büsgen ('91) that certain modifications are produced in the tissues of the food plants of Coccids at the point where the mouth setæ are thrust into the plant. These modifications appear to be induced by secretions actually injected into the plant tissue by the insects and they may act in liquefying or in partly digesting already liquid or semi-liquid material, before it is withdrawn by the insect. It is quite possible therefore that a diastatic ferment might act in two possible ways in aiding the digestion of the coccid. If freed in the blood, it might either pass into the alimentary tract, there to act upon ingested food, or it might be taken up by the salivary glands to be later injected into the plant and thus act as an extra-intestinal digestive agent. Such extra-intestinal digestion is known to occur in several diverse insects, although in these cases the ferments are no doubt elaborated directly by the salivary glands.

We have also clearly shown that a proteolytic enzyme and a lipase are produced abundantly by the *Pulvinaria* symbiont. The possibilities for these to influence the digestion and metabolism of the coccid are more diverse than those presented by the presence of diastase. Both, particularly the lipase, must act upon the adipose cells in which the symbionts occur. We might therefore suppose that they assist in the rapid breaking down of this tissue at the time of maturity when the eggs of the *Pulvinaria* are rapidly developed. That they may assist in digestion, either in the body or through the agency of secretions injected into the plant is also quite possible, although such indirect action must undoubtedly be secondary if it occurs at all.

The General Nature of the Relation between Symbiont and Coccid.

The symbionts have gradualy come to be regarded rather generally as truly symbiotic organisms, although those who first studied them naturally assumed that their presence indicated some sort of parasitism. There are several reasons why it is difficult to believe that they are actually parasitic. In the first place, not only in *Pulvinaria,* but in the other species in which they have been found, they are universally present in all the individuals of a species in approximately equal numbers. Many true parasites, *e.g.,* certain Nematode worms, the Protozoan parasites of human malaria, etc., commonly appear with great frequency in the bodies of their hosts, but their occurrence never includes all the individuals of a host species, except at certain times and places where parasitism is unusually heavy and assumes the form of an epidemic. In such cases also, the affected population is not in a healthy condition and species so generally affected cannot be expected to represent ones well fitted to survive and become abundant. *Pulvinaria* and other Coccids certainly cannot be placed in such a category. On the other hand the presence of detrimental parasites results in tissue changes or disturbances of metabolism that can be recognized. Such can be seen in the behavior of the fat-body in *Pulvinaria* and other genera (Sulc, '11), but as has just been said this is most readily regarded as beneficial rather

than detrimental as the fat is not broken down until the time that it would normally disintegrate to supply nourishment for the developing eggs of the coccids.

Without any definite indication of pathological changes, it seems impossible, therefore, to regard the universally present symbionts as harmful parasites.

It has also been suggested that they may represent innocuous or indifferent parasites and it is not so easy to distinguish between these and true symbiotic or benign organisms from their effect on the coccids. As a matter of fact it seems necessary to regard all three as steps in an evolutionary process, harmful parasites in their first association, later as innocuous ones and finally as true symbionts. These will follow one another as the host adapts itself to withstand or nullify any ill effects of the parasite until it finally is able to utilize the products of the intruder to further its own metabolic processes.

Thus it seems reasonable to regard these three types of association as not clearly distinct from one another, but as connected by intergrades.

Since, however, there is good reason to believe that the production of diastase, protease and lipase by the symbionts may serve to benefit the coccids, the possibility of real symbiosis cannot be excluded.

There is one point, however, which needs further study. By a minute study of the changes in the tissue of the food-plant adjacent to the proboscis of the feeding Coccid, it should be possible to gain much additional evidences upon the changes which undoubtedly occur in such tissue. This we have not had opportunity to undertake. Why the disintegration of the fat-body is delayed till the proper time in the life-cycle of the coccid also is not clear. Since, however, changes in the vegetative character of the symbiotic fungus are initiated in the late spring, it seems probable that they may determine to some extent the quantities of enzymes produced. On the other hand it is evident that the coccid is able to inhibit any excess development of the symbiont as the number of symbiont cells remains very uniform and never seems to increase beyond certain bounds, quite a different condi-

tion from that obtained among pathogenic parasitic microörganisms.[1]

This indicates a nice physiological balance between the coccid and symbiont and is another reason for considering this a case of true mutualism.

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EXPLANATION OF PLATES.

Plate I.

1. Cross-section of overwintering Coccid, showing distribution of symbionts in the fat-body. Low magnification.

2. Portion of fat-body at higher magnification with symbionts included in adipose cells. Magnification about 400 diameters.

3. Similar portion, showing more abundant symbionts. Magnification about 400 diameters.

4. Symbionts in adipose tissue which is apparently in process of disintegration. Magnification about 400 diameters.

5. Smear from culture of symbionts in liquid medium after 48 hours incubation. Magnification about 400 diameters.

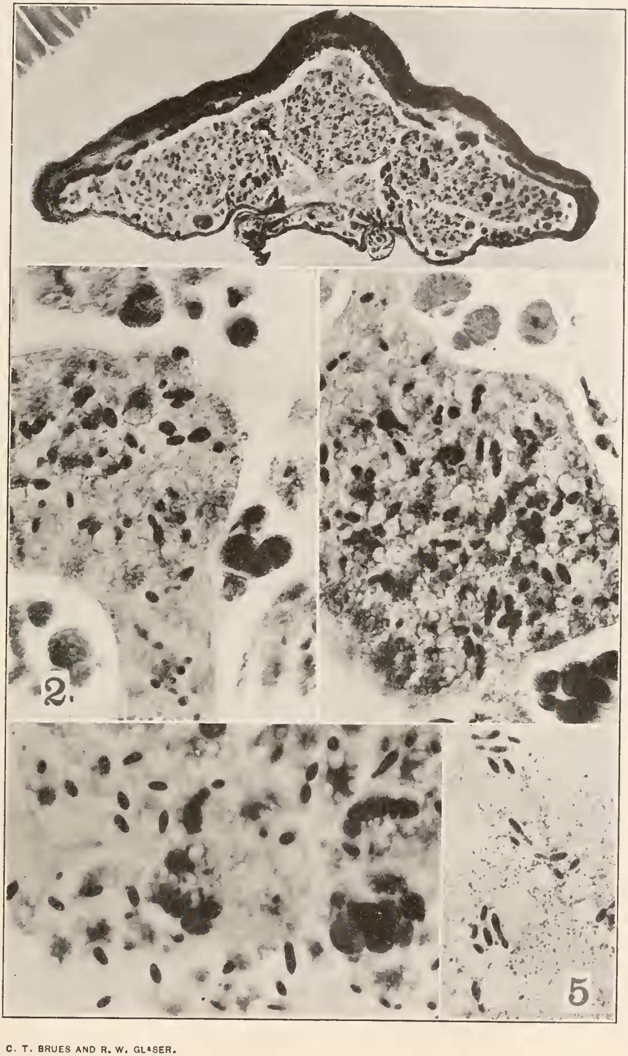

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Plate II.

6. Form of young colonies of symbiont after 72 hours incubation on potato agar plate, viewed by reflected light. Magnified 5 diameters.

7. Group of colonies of symbiont on nutrient agar after 10 days incubation. Magnified 4 diameters. Note small size of colonies and paucity of processes.

8. Group of colonies on the plate illustrated in figure 6, at same magnification, viewed by transmitted light, to show internal structure and manner in which radial processes develop.

9. Development of mycelia in liquid culture of symbiont after prolonged incubation of 10 days. Magnified about 60 diameters.

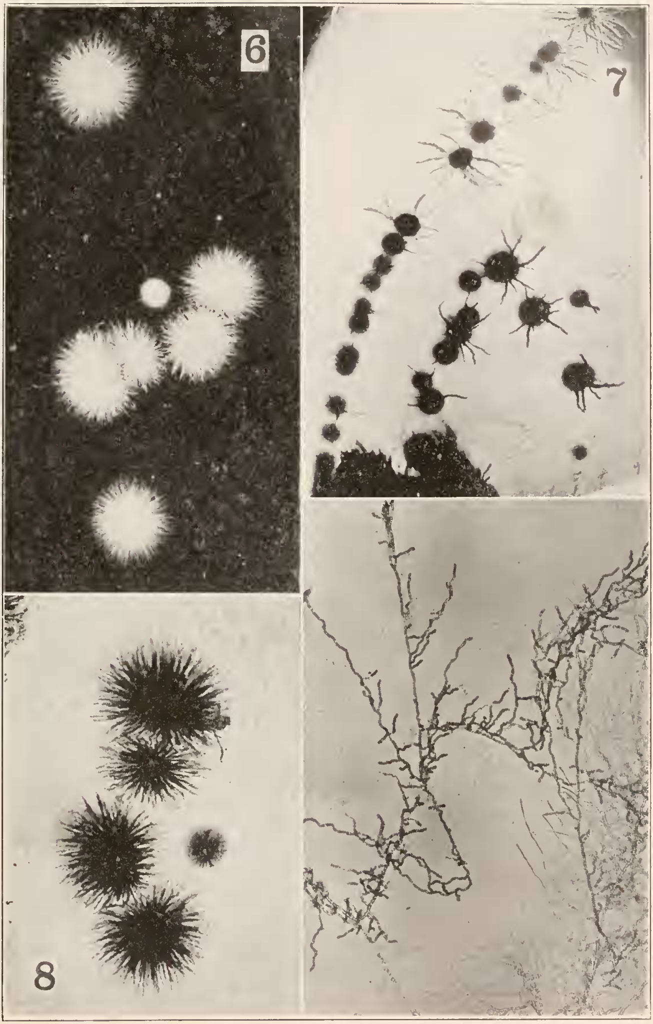

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PLATE III.

10. Gross appearance of colonies of symbiont after prolonged incubation (12 days) on potato agar. Viewed by reflected light to show peripheral processes. Magnified 3 diameters.

11. Portion of same plate at same magnification, viewed by transmitted light, to show internal mycelial structure.

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THE MIGRATION OF THE PRIMARY SEX-CELLS OF FUNDULUS HETEROCLITUS.

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The origin of the primary sex-cells in vertebrates is a problem which has received considerable attention during late years. Extensive summaries of the literature upon this subject may be found in the articles of Allen, 1911, and Jordan, 1917. Since no complete agreement with regard to details has as yet been reached, it is perhaps desirable to review briefly the earlier investigations, and to point out any discrepancies in the results already obtained which would seem to require further study. It would seem that more evidence is necessary to warrant safe conclusions on the matter.

Waldeyer (1870) first described the differentiation of the sex-cells from the "germinal epithelium" of a four-day chick. His view of sex-cell origin from the mesothelium covering the mesonephros was accepted at the time, and has been supported even by recent investigators. In 1880 Nussbaum advanced a rival theory as a result of his observations on the embryology of the trout and frog. He held that the sex-cells were of blastomeric origin, and further that there was an extra-regional segregation and a migration to the germ gland. Weismann (1886) popularized this idea in his work on the "continuity of the germ plasm."

Since the time of Nussbaum the evidence against the "germinal epithelium" idea has steadily increased. A number of investigators (Hoffman, 1892; Eigenmann, 1892; Beard, 1900; Woods, 1902; Allen, 1906, 1907, 1911; Dodds, 1910; Swift, 1914, 1915, 1916; Jordan, 1917) have failed to find any conditions not in accord with Nussbaum's theory.

However other recent workers (Firket, 1914, 1920; von Berenberg-Gossler, 1914) have been unable to accept this interpretation of the activities of these cells. According to their viewpoint, the

migration of the primary sex-cells is reduced to a mere phylogenetic vestige and is without any great genetic significance. Firket speaks of the primordial germ-cells as "primary genital cells" which, after migration disintegrate in the germ gland, being replaced by the true of "secondary genital cells" which arise from the peritoneal cells of the germ gland. Von Berenberg-Gossler regards them as mesodermal wandering cells of late endodermal origin, and describes them as contributory in the formation of the Wolffian ducts.

In our study of this general problem in *Fundulus* a number of questions have arisen as separate phases of the matter. The blastomeric origin of the sex-cells, their path and method of migration, and their history after reaching the germ gland are all matters requiring separate study. This paper has, as its special aim, the definite identification of the primary sex-cells and the determination of the germinal path in *Fundulus* embryos; that is, it is concerned with the second question listed. As yet our data upon the first question is inadequate and we have not enough material for a study of the third.

Material and Methods.

The material for this investigation consisted of the eggs of the teleost, *Fundulus heteroclitus* and was collected at Woods Hole in the summer of 1919. Care was exercised to insure an approximately uniform fertilization of the ova by mixing them with chopped testis. Two extensive series were preserved during the summer. Although accurate records were kept as to the age of each group, they are of only nominal value in this investigation, since environmental and individual differences cause variations in development of embryos of like age.

All embryos in these two series were fixed in Bouin's fluid and stained by the familiar "long method" for iron hæmatoxylin. Other material in various fixatives was also available for comparison. No trouble was experienced in obtaining slides which show clearly the cytological characteristics throughout the series as far as described. A majority of sections were cut 4 micra thick, but some were cut 5, 6 and 7 micra. The thickness of all sections

was of course recorded. Most of the observations were made from serial transverse sections because they show the dorsoventral position of the sex-cells more clearly in relation to the outstanding features of the developing embryo than do those cut longitudinally.

Observations.

Criteria of the Primary Sex-cells.

The enumeration of criteria for any group of cells as distinguished from all others in a series of embryos is a task which promises but doubtful results. There can be no question however that the primary sex-cells do have distinctive characteristics which make them easily recognizable, during the resting stages, to one who has had them under observation. It is not always feasible positively to identify the cells during division.

Throughout the migration period these cells maintain the same general characteristics. There are, to be sure, slight variations in the ratio between the nuclear and cytoplasmic elements, in size and in the character and arrangement of the chromatin granules; but these features may be observed only on close inspection, rather than in a preliminary study of the primary sex-cells.

The primary sex-cells vary in diameter from 9 to 12.8 micra. As contrasted with other cells they are spherical or ovoid with very definite cell outlines. The nuclei conform to the general shape of the cell body within which they are located. The cytoplasmic content is always clearer and takes less stain than that of the surrounding cells. Likewise the achromatin of the nuclei is quite clear, allowing the chromatic granules to stand out in bold contrast. The linin network is directly beneath the nuclear membrane, and due to this arrangement the chromatin granules are distributed peripherally over the nucleus. This peripheral arrangement of the chromatin is a constant distinguishing characteristic not to be mistaken, for it is never produced in any other cells. The linin network is connected to one, or more frequently to two nucleoli which are located near the center of the nucleus. No peculiar invagination of the nuclear membrane, such as was reported by Dodds (1910), was observed in *Fundulus*. An unusually large centrosome is, as a rule plainly visible in the cyto-

plasm. In older embryos these cells may be recognized by their size, since they are larger than any others which may occur in the same region.

Figures 1, 2, 3 and *3a* are surface views of typical sex-cells. The peripheral arrangement of the chromatin has been emphasized in drawing Fig. *4b*, by focusing upon a level with the center of the nucleus. Fig. *4a* was obtained by focusing higher on the surface of the same nucleus. Thus Fig. *4b* represents the chromatin knots in an optical section; while Fig. *4a* shows them in a surface view. If Fig. *4a* were superimposed upon Fig. *4b* the resulting composite would be a cell not unlike that represented in Fig. 3, except that in the latter the knots have taken the familiar granular appearance.

A positive identification of the primary sex-cells was first made in a 24-day embryo. From this stage their path was followed backwards, through all the intermediate phases of migration, until they were no longer evident. It is considered expedient to describe their position in the 24-day stage, so that no question shall arise later as to the exact nature of the migrating cells whose course is to be traced.

Having established the identification of the sex-cells in the late embryo (24 days) their migration may be traced from their earliest appearance up to this stage. Although this sequence is contrary to our experimental procedure, it is believed to be more easily followed by the reader.

24-Day Embryo. 5.75 Mm. Long.—At the 24-day stage the sex-cells lie in the sac-like anlagen of the germ glands, which have formed dorsally and slightly laterally to the hind gut. Here they are unquestionably recognizable (Fig. 5). These cells are numerically inferior to the peritoneal cells which surround them and which are beginning to take a very active part in the formation of the future sex gland. The size and position of the germ gland anlagen in relation to the embryo is shown in Figs. 6 and 7. No attempt has been made to ascertain the average number of sex-cells which are present during this stage.

Whether these are the true sex-cells as maintained by many investigators, or whether they later disintegrate and become replaced by "secondary genital cells" as indicated by Firket (1914,

1920) and others, is a question which may be omitted from the present discussion.

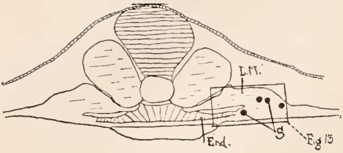
Observations of the Germinal Path.

From many available embryos the following were selected for consideration because they constitute representative stages, and are essential to a clear understanding of the migration of the primary sex-cells.

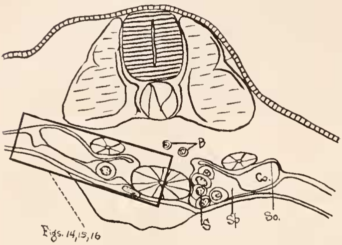
Embryos from 46 to 50 Hours.—Three embryos of this very early stage, designated in our material as B′ 21X, B′ 21L and B′ 23 respectively were carefully studied. Others were available but they were used merely as checks on the three which are reported.

The position of the sex-cells at this stage is most striking. There is a wide range of distribution in each embryo. The most anterior of the sex-cells were invariably farther along the germinal path than were the more posterior ones of the same embryo. To demonstrate this fact Fig. 20 has been drawn; it is an exact outline diagram of B′ 21X, reconstructed by the most accurate means possible. The lateral extent of the neural tube, of the mesoderm and the positions of the sex-cells were determined by measuring from the median line. The thickness of the sections was known. These determinations were plotted on millimeter paper and the outline filled in as indicated by the plotted guides. The exact antero-posterior positions of the sex-cells were determined by counting the sections of the serially sectioned embryo. Figs. 9, 10 and 11 are outline drawings from the embryo B′ 21X which was sectioned transversely, and Fig. 12 from B′ 21L which was sectioned longitudinally. These drawings show the exact positions of the sex-cells more clearly than would be possible in a written description.

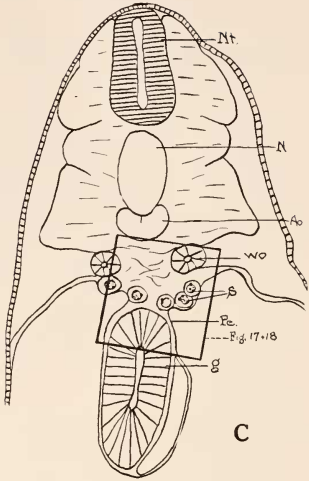
The letters *A, B* and *C* on Fig. 20 indicate the positions of the sex-cells shown in Figs. 11, 10 and 9 respectively. Fig. 11 illustrates clearly the position of the primary sex-cell in the extra-embryonic region at the posterior of the embryo. Four sex-cells are shown in Fig. 12 as being lateral to the undifferentiated endodermal cell mass and ventral to the elongating tail. In Figs. 9 and 10 the migration has progressed proportionally to the devel-



A



B



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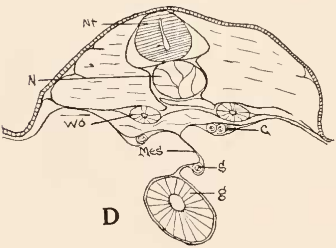

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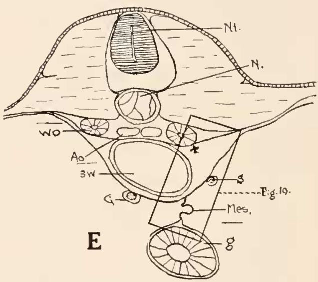

E

TEXT FIG. *A*. Semidiagrammatic transection through the posterior of the 50-hour embryo B′ 23. The spots indicate the positions in which the greater portion of the sex-cells are found at this stage. The rectangle includes the area which is drawn in detail in Fig. 8. × 225.

TEXT FIG. *B*. Transection from the 105-hour embryo, showing the first decided advance over the stage illustrated in Text Fig. *A*. Here the gut, Wolffian ducts and the cœlome have taken form. The rectangle includes the area drawn in Figs. 14, 15 and 16. × 225.

TEXT FIG. *C*. Showing progress of development after 6 days. Figs. 17 and 18 are detailed drawings of the area included in the rectangle. × 225.

TEXT FIG. *D*. Transection through the developing gonads of the 9-day embryo, B 34. The sex-cells are collected ventral to the Wolffian ducts. The dorsal mesentery shows a decided change from conditions found in earlier stages. × 90.

TEXT FIG. *E*. From the 13-day embryo B 42, showing the effect of the developing swim bladder. × 90.

opment of the embryo at the regions represented. The sex-cells lie between the periblast and the endoderm in Fig. 10; while in Fig. 9 their position is below the mesoderm and lateral to the developing hind gut.

The positions of the most anterior sex-cells in embryo B′ 23 are indicated in Text Fig. *A*. The rectangle in this text figure includes the region which is drawn in detail in Fig. 8. Here a primary sex-cell is shown which is entirely free from any possible connection with the lateral mesoderm. It can scarcely be said to lie in, but rather lateral to the gut endoderm. It is half buried in the periblast. This fact suggests intimate relation with this nutritive layer. The cell figured is one of the few ever found with an irregular outline. This might seem to suggest amœboid activity, but this type is so extremely rare that it may be neglected from consideration.

Fig. 13 from B′ 23 shows a sex-cell which is .06 mm. to the rear of the one just mentioned. It is plainly in that portion of the lateral mesoderm which will develop into the splanchnic layer upon the formation of the cœlome (about the third day).

Observations of these early embryos show several important facts. The primary sex-cells are as truly characteristic and as easily recognizable as any found in the germ glands of later stages. They are located in the posterior half of the embryo, becoming gradually more numerous as the anterior part of this region is approached. Laterally they range from the extra-embryonic region to within the lateral mesoderm and the edge of the developing gut. In general their progress along the germinal path is directly proportional to the development of the embryo.

105-Hour Embryo.—Text Fig. *B* shows the relative positions of the sex-cells in the 105-hour embryo, B′ 26. As in previous cases the rectangle indicates the area from which Figs. 14, 15 and 16 were drawn. These three figures from the same embryo illustrate the full extent of the migration at this stage. On the left side of the embryo the sex-cells are found scattered all along the splanchnic mesoderm, from the region very near the split in the lateral mesoderm (Fig. 14) to that at the side of the gut (Fig. 16). On the right of Text Fig. *B* the sex-cells on the opposite side of the embryo are shown massed lateral to the gut. Should

the lateral mesoderm fuse above the gut, the formation of the dorsal mesentery would result and the position of the sex-cells would be identical to that found in later stages. Ex. Figs. 17 and 18.

6-Day Embryo. 2.6 Mm. Long.—Text Fig. *C* represents the position of the sex-cells as found in the 6-day embryo. At this time they are apparently in a state of rapid migration from the loose mesenchyme dorsal to the hind gut, to the positions ventral to the Wolffian ducts. Because of the laterally compressed condition of the embryo, which was due to the softness of the paraffin at the time of cutting, the transections are not exactly typical. However this embryo has been used since it represents most clearly the transitional stage between those figured in Text Fig. *B* and *D*. Figs. 17 and 18 are detailed drawings of the 6-day stage. They illustrate the complete extent of the migration in the mesentery. The majority of the sex-cells were in the dorso-ventral position indicated by the cells in Fig. 17, while only a few were in that shown in Fig. 18. The more anterior sex-cells were farther along in the germinal path (being nearer the Wolffian ducts) than the more posterior ones. The position of the germ gland anlagen ventral to the Wolffian ducts is illustrated in Text Fig. *D*.

13-Day Embryo. 4 Mm. Long.—Text Fig. *E* represents the position of the germ gland anlagen as found in the 13-day embryo B 42 (4 mm.). The rectangle in Text Fig. *E* includes the region which is drawn in detail in Fig. 19. Rarely more than one sex-cell is found at this stage in any one section of a germ gland anlage. The anlagen are little more than protuberances from the peritoneum, containing relatively few peritoneal cells (although the sex-cells are surrounded by them) and they have not yet reached the future position of the gonads. It is obvious that the sex-cells which are contained in the peritoneal sac are all pushed ventrally by the developing swim bladder. One cell was observed in the position indicated by the cross in Text Fig. *E*. It was not included in the peritoneal sac and seemed apparently helpless in the loose mesenchyme ventral to the Wolffian duct. This cell had been delayed in reaching this position, had not been included in the sac, and in consequence of this fact it had not been

influenced by the action of the swim bladder. One of these lost cells is shown in the mesentery in Text Fig. *D*. The future of such cells is an open question.

A count of the sex-cells in this embryo, B 42, gave 64. There was never any question of recognizing these cells, for no cells of doubtful character were observed. Four of these cells found were in the mesentery above the gut, and one was in the loose tissue ventral to the Wolffian duct. No cases of disintegrating sex-cells were observed in our *Fundulus* material, although such conditions are reported by some investigators.

The *24-day embryo* shows the next advance in the germinal path. This stage has been considered perviously in connection with "Criteria of the Primary Sex-Cells."

Table of Average Diameters.

For the purposes of this investigation the diameters in micra were found by averaging the long and short dimensions of the cell and nucleus. By this method a number of representative sex-cells of embryos in all stages of migration were measured under the oil immersion and the following results were obtained.

Embryo.								Average.
1.B′21X (embryonic region) 46 hours	Cell	10.0	10.5	9.6	10.4			10.1
	Nucleus	6.0	6.4	5.6	5.2			5.8
2.B′21X (extra-embryonic) 46 hours	Cell	12.8	11.2	11.2	11.5			11.7
	Nucleus	6.0	6.1	6.5	6.5			6.3
3.B′26 105 hours	Cell	9.9	11.0	10.7	10.4	10.2		10.4
	Nucleus	6.1	6.2	6.2	6.1	5.8		6.1
4.B 30 6 days	Cell	12.3	12.4	12.7	11.2			12.2
	Nucleus	6.6	6.6	7.0	6.2			6.6
5.B 42 13 days	Cell	11.5	11.5	11.9	10.7	9.9	11.5	11.2
	Nucleus	7.2	6.6	8.0	7.8	5.7	6.3	6.9
6.B 65 24 days	Cell	11.5	11.8	10.2	10.4	9.0	9.5	10.4
	Nucleus	6.1	7.8	7.3	7.0	6.2	6.6	7.2

Multiplication of the Sex-cells.

The early distribution of the sex-cells (Figs. 20 and 21) is best explained, we believe, in connection with the streaming of the organ-forming substances which contribute materials to the embryo body. In most processes of this nature not only cell trans-

portation but cell division takes part. Certain workers with other forms have held that the movement of the cells into the anlagen of the gonads is not the only factor responsible for their increase, but that multiplication actually occurs during the period of translocation. Mitotic figures have never been observed in *Fundulus* among the recognizable sex-cells which are within the embryo, although a most thorough search has been made for them in many embryos at all stages of development. A count of these cells in several specimens in various stages reveals the fact that there is a tendency for their number to vary more or less from the average established (67). However there is not enough variation to convince one that there is any marked multiplication of the sex-cells during the migration period. These facts naturally lead to the conclusion that the first period of multiplication takes place in the extra-embryonic region.

In the description of the earliest embryos referred to in this report and in the figures presented, emphasis has been placed upon the fact that the primary sex-cells in any one embryo are not in the same phase of migration. Furthermore observations upon all stages show that development becomes more advanced anteriorly than posteriorly. It is of further interest that in embryos containing sex-cells both within and without the body, the number falls below the average for older stages. These conditions and the fact that no sex-cells have been found in any region other than that already described, suggest the explanation that these cells multiply in the extra-embryonic region. Indeed the four sex-cells illustrated in Fig. 12 may indicate recent cell division by their very association. If they are not of recent and identical origin they would probably be farther separated than they are in this figure. These views are presented only tentatively, due to lack of sufficient material to warrant definite statements on this multiplication; for no mitotic figures have ever been seen in the extra-embryonic region to substantiate this belief in a division as suggested. Our material has not permitted a careful study of this matter. However considering the longitudinal distribution of the sex-cells in the earliest available embryos, and their tendency to approach a common average number in each individual, one is inclined to regard them as being of unquestionably

earlier origin than it has been possible thus far to trace them. It seems not unreasonable to believe that the fore-runners of these cells have been segregated at a time very early in the development of the germ ring.

Discussion and Conclusions.

This paper attempts to identify definitely the sex-cells which are present in the 24-day embryo as the "primordial germ cells" of previous writers, or as the "primary genital cells" of Firket. It also presents evidence on the manner in which these cells reach their final destination.

The method of embryo formation in the teleosts has a bearing upon the question of sex-cell migration in *Fundulus*. It will be recalled that the anterior portion of the embryo is formed from the head fold, which may perhaps be nothing more than a thickening on the germ ring; while the body or posterior portion is to be regarded as the result of the developmental process termed concrescence. It is only this latter portion of the body that is involved in the formation of the sex-cells. The eggs have a large amount of yolk, and a very distinct germ ring. As cell proliferation takes place, the germ ring moves gradually downward over the yoke mass. The primitive streak moves backward and receives the converging limbs of the germ ring posteriorly. The material of the halves of the germ ring, after fusion, is differentiated into the embryo posterior to the head process. The rudiments of the embryo body are not clearly marked out in *Fundulus* until the germ ring is completely closed.

The earliest primary sex-cells which we have located are from embryos in which the germ ring has been closed but a few hours, and in which the tail is just beginning to elongate. Their position in the extra-embryonic region lateral to the undifferentiated endodermal cell mass at the posterior half of the embryo is indicated in Fig. 20. In other embryos of the same stage of development, numerous primary sex-cells are present in practically the identical relation to the embryo that is clearly demonstrated in Fig. 20. These sex-cells invariably lie just above the periblast and are associated with the sheet of cells which is a lateral expansion of the undifferentiated endodermal cell mass

(peripheral endoderm, Allen). The complete germinal path from this position to one lateral to the hind gut may be followed in almost any embryo of from 46 to 50 hours. This very advantageous condition is made possible by the greater development near the middle of the embryo, for it is only a natural result of embryo formation by concrescence that development is progressively greater anteriorly from the point of convergence of the germ ring.

These cells are transported from the edge of the embryonic region medially, to positions just beneath or within the endodermal cell mass, as the case may be. They are carried passively from one position to another by the same forces of growth which bring together the halves of the germ ring. The influence of this factor can scarcely be over emphasized. Although not outwardly as apparent as in earlier stages, these forces are nevertheless responsible for the flowing of the streams of embryonic material towards the future position of the organs which are to develop therefrom.

The sex-cells come to lie within these shifting layers of embryonic endoderm and mesoderm and naturally accompany these layers in their changes of position. Because of the fact that the movement of the sex-cells is not active but rather dependent upon that of surrounding layers, the expression "migration" seems rather unfortunate. Some term such as "translocation" would perhaps be more truly expressive of the actual conditions.

These cells come to lie in the edge of the embryonic region, and when a portion of the undifferentiated cell mass gives rise to gut endoderm and another to lateral mesoderm, they follow one layer or the other. The sex-cells follow one or the other of these layers until they reach a position lateral to the newly formed gut. Which layer is chosen apparently depends upon chance. Those cells which have been carried in the edge of the endoderm never enter the gut, but move dorsally from the side of it into the lateral mesoderm. Here they join the sex-cells which have been carried in the mesoderm. By this time the split, resulting in the formation of the cœlome between the splanchnic and somatic mesoderm has taken place. Although the sex-cells are associated with all parts of the lateral mesoderm before the forma-

tion of the cœlome, it is a noteworthy fact that they never occur within the somatic layer after differentiation.

From this position lateral to the hind gut the cells are in the general dorsal movement of the mesoderm which eventually results in the formation of the intestinal mesentery. The cells from either half of the embryo remain apart and seem to lie in separate streams of mesodermal cells which are flowing toward the Wolffian ducts. But although there may be a pause here, at no time do the sex-cells appear to establish any intimate relation with the cells of these ducts (Text Fig. *D*). From the evidence at hand, an explanation of the function of these cells which makes them contributory to the development of the already well-formed Wolffian ducts, as suggested by certain investigators, does not seem plausible in *Fundulus*.

As the sex-cells reach a position nearly ventral to the Wolffian ducts they become surrounded by a single layer of peritoneal cells. This covering develops until the position of the future sex organs is attained; the sex-cells then rest in sac-like protuberances from the peritoneum, the germ gland anlagen. Assisting in the movement which brings the sex-cells into their future positions, are several factors entirely external to the germ glands. For example, there is a rapid proliferation of the loose mesenchyme dorsal to the gut and the development of the swim bladder which results in a median down pushing. The ventral movement (Text Figs. *D* and *E*) from the region of the Wolffian ducts is clearly due to the wedge-like effect produced by the growing swim bladder. That this process is necessarily passive is evident from the fact that amœboid activity of the cells included within the germ gland would be unable to produce any change in its position.

From the evidence in *Fundulus* it is apparent that the sex-cells enter the embryo and are located in the germ glands by the same forces that are influential in the distribution of the other organ forming substances of the body. Their "migration" is not to be looked upon as different from that of any other group of cells. But while the sex-cells are not amœboid, there is nevertheless reason for misunderstandings which have arisen regarding their activities. In the first place they are relatively few in compari-

son to the great numbers of cells in the surrounding tissues. Although the entire mass of cells is continuously in motion, only the movement of the sex-cells is at all noticeable. They are shifted about by the active surrounding tissues and naturally assume slightly irregular outlines at times, due to the unequal tension upon the cell membrane. Through a misinterpretation of the conditions within the embryo these sex-cells may easily be accredited with peculiar powers of locomotion. A sex-cell, as a slowly drifting cloud, can be seen gradually to change its position; but movements of the tissue cells about it, due to their location in continuous layers, are so inconspicuous as to go unnoticed. Because of this, the movement of the sex-cells should be considered merely as the passive indication of the rate and direction of progress of contiguous layers.

Summary.

1. The earliest primary sex-cells found in *Fundulus* were located in the peripheral endoderm, lateral to the posterior half of the 46-hour embryo. No sex-cells were observed in that part of the embryo which develops from the head fold.

2. The germinal path leads from the peripheral endoderm, into the border of the undifferentiated endodermal cell mass. When this cell mass splits to form gut endoderm and lateral mesoderm, the sex-cells proceed medially with either layer. By the time the gut is formed, these cells are lateral to it; they all eventually become located in the splanchnic mesoderm of this region. From here the sex-cells migrate dorsal to the hind gut, thence to the region ventral to the Wolffian ducts. Here they become surrounded by peritoneal cells which form the somatic portion of the gonads. From this position the germ gland anlagen are shifted back to their final location dorsal to the gut.

3. There is very little multiplication of the sex-cells during the period of migration. Division apparently takes place in the extra-embryonic area, and is not renewed to any marked extent until after the sex-cells become located in the germ glands.

4. The constant distinguishing characteristics insure positive identification of these cells throughout all phases of their migration, and leave no reason to question their identity as being the "primordial germ cells" of previous writers.

5. Migration is passive, being due to forces of growth which are altogether external to the cells themselves. These forces of growth are factors common to the development of the organs formed in the body of the teleost embryo.

6. Evidence derived from this study of *Fundulus* is an absolute harmony with the theory of early segregation of these primary sex-cells.

Note

Some time after the manuscript of this paper had been sent to the press, an extensive article by Okkelberg, entitled, "The Early History of the Germ Cells in the Brook Lamprey, *Entosphenus wilderi* (Gage), up to and Including the Period of Sex Differentiation," appeared (*Jour. Morph.*, Vol. 35, No. 1, 1921). This article contains much data and many important conclusions, and it is to be noted (on pages 35 and 36) that the author, in considering the sex cells, has discussed their methods of migration. It is of great interest that the conclusions reached by Okkelberg on this matter for the lamprey are very similar to our own upon *Fundulus*.

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EXPLANATION OF ILLUSTRATIONS.

All figures in this report were drawn with the aid of a camera lucida. Any lens combinations which were considered necessary to produce the best results were used. The magnification as given for each figure was calculated carefully and is correct for the reproductions as they appear on these plates.

ABBREVIATIONS.

a., anus.
Ao., aorta.
B., blood cells.
c., centrosome.
cr., chromatin knots.
Co., cœlome.
Ect., ectoderm.
E.M., endodermal cell mass.
En., gut endoderm.
G., germ gland anlagen.
g., gut.
L.M., lateral mesoderm.
li., linin network.
M., mesoderm.
Mes., gut mesentery.
N, notochord.
Nt., neural tube.
nu., nucleolus.
P., peritoneum.
pe., periblast.
P.N., periblast nucleus.
S., sex-cell.
sw., swim bladder.
So., somatic mesoderm.
Sp., splanchnic mesoderm
T., elongating tail.
Wo., Wolffian duct.

DESCRIPTION OF ILLUSTRATIONS.

PLATE I.

FIG. 1. A typical primary sex-cell from a 105-hour embryo (B′ 26). Showing the large centrosome, two nucleoli and chromatin knots scattered over the periphery of the nucleus. × 1420.

FIG. 2. A typical sex-cell from a 6-day embryo (B 30). Chromatin granules finer than in the preceding cell. × 1420.

FIG. 3. Sex-cell from a 100-hour embryo (B′ 25–3). From the extra-embryonic region. It is closely associated with the periblast. In other sections the peripheral endoderm may be seen out over this area. × 1420.

FIG. 3*a*. Sex-cell from a 9-day embryo (B 34). Chromatin arrangement is intermediate between that found in Figs. 1 and 2. × 1420.

FIG. 4*a*. Surface view of a nucleus from a sex-cell in a 105-hour embryo, showing the chromatin knots in surface view. × 1420.

FIG. 4*b*. From the same nucleus as the one used in Fig. 4*a*. Obtained by focusing upon the center of the nucleus, illustrating the chromatin knots in optical section. × 1420.

FIG. 5. Showing the sex-cells in the germ gland anlagen dorsal to the hind gut of a 24-day embryo. The function of the peritoneal cells at this stage is quite evident in this illustration. The sex-cells are completely surrounded by mesoderm. × 700.

FIG. 6. Transection through a 24-day embryo (B 65–2) taken at the position indicated by the plane X–X′ in Fig. 7. Showing the position of the gonads in relation to other parts of the embryo; especially as regards the developing swim bladder. × 120.

FIG. 7. Longitudinal section lateral to the median line of the 24-day embryo (B 65). Illustrating the position of the gonads as being the same as in the adult. Migration ceases at this point. The activities within the gonads after they have reached this point of development will not be considered at this time. × 25.

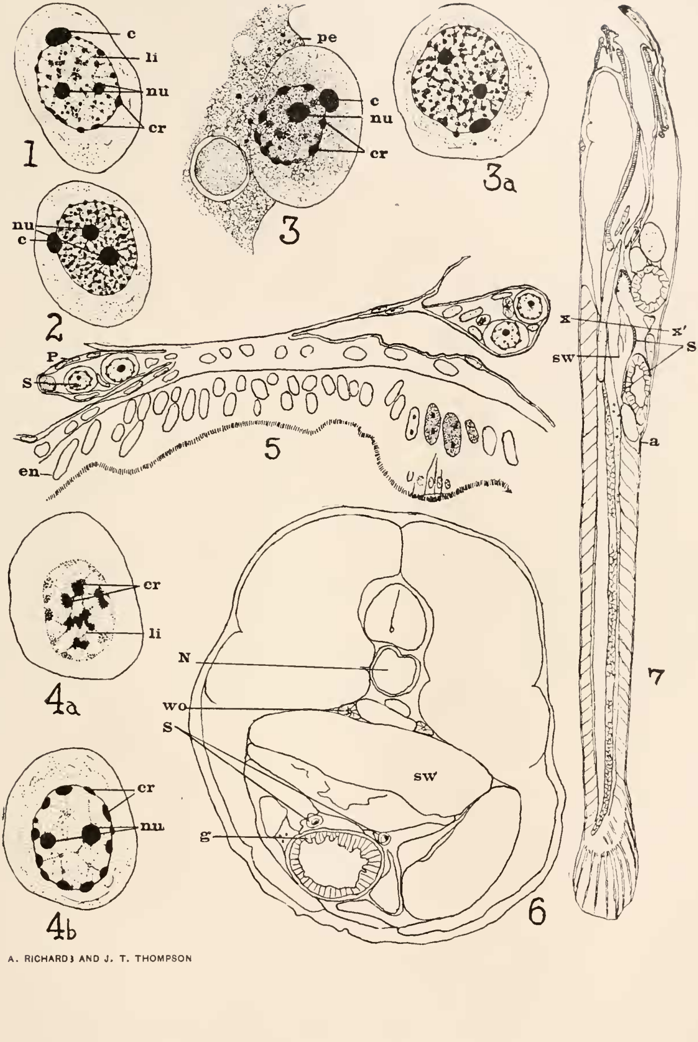

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Plate II.

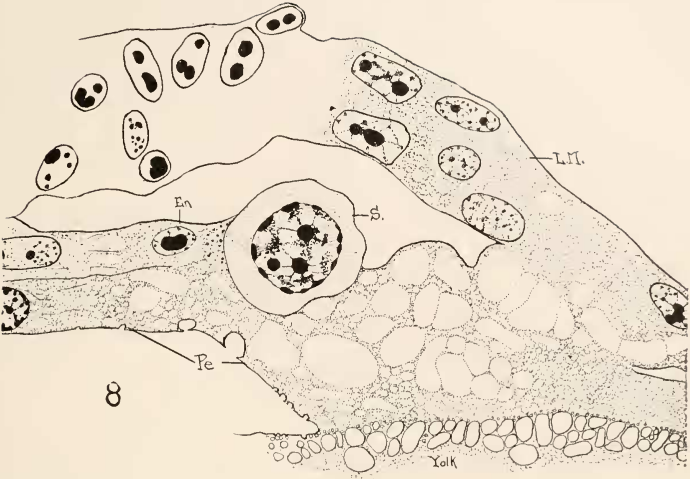
Fig. 8. A sex-cell from the 50-hour embryo (B′ 23). Showing in detail the region at the edge of the embryo, where the germ layers meet the periblast. The sex-cell is in the edge of the gut endoderm, entirely removed from any connection with the lateral mesoderm, and is partially imbedded in the periblast. Several very early cells have been found in this relation to the periblast, but so far it has been impossible to establish any significance to this fact. Due to exertion of unequal tension upon the cell membrane, the cell outline appears slightly irregular. × 1400.

Fig. 9. Semi-diagrammatic transection of the 46-hour embryo B′ 21 X taken at the position indicated by the line "*C*" in Fig. 20. At this early stage the lumen of the gut is not formed completely, even in this most anterior region. The sex-cells lie between the periblast and the lateral mesoderm, at the side of the developing hind gut. × 300. (The positions of the sex-cells in the germinal path correspond to certain stages in development of the gut.)

Fig. 10. Transection of embryo B′ 21 X taken at the position indicated by the line *B* in Fig. 20. Here the gut endoderm and the lateral mesoderm are differentiated to a certain extent, although the splitting of the endodermal cell mass has not yet occurred. The sex-cell lies above the periblast in the edge of the cell mass. × 300.

Fig. 11. Transection of embryo B′ 21 X taken at the line "*A*" in Fig. 20. The sex-cell illustrated is in the extra-embryonic region, associated closely with the peripheral endoderm. × 300.

Fig. 12. A longitudinal section through the elongating tail of the 46-hour embryo B′ 21 L. The 4 sex-cells illustrated are in the peripheral endoderm at the extreme posterior of the embryonic area. × 300.



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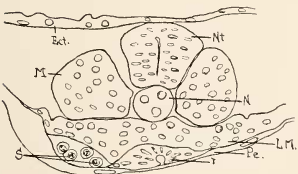

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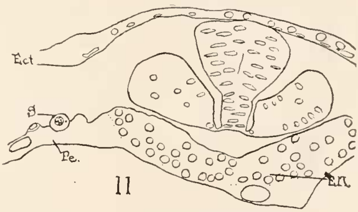

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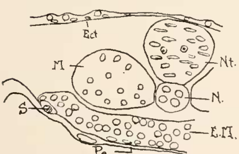

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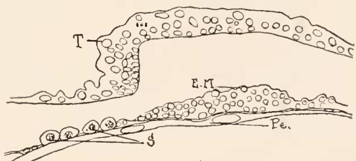

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Plate III.

Fig. 13. Sex-cell from the 50-hour embryo B′ 23. It lies in the extreme edge of the lateral mesoderm, just dorsal to its separation from the periblast. This cell lies in that portion of the mesoderm which will give rise to the splanchnic layer. × 700.

Fig. 14. A sex-cell in the splanchnic mesoderm of the 105-hour embryo B′ 26. The cell is just medial to the point of differentiation between the somatic and splanchnic layers. This stage also shows an advance over the one illustrated in Fig. 13, in that the gut and Wolffian duct have taken form. × 700.

Fig. 15. A group of sex-cells from the region anterior to that drawn in Fig. 14. Here the sex-cells are approaching the side of the hind gut. × 700.

Fig. 16. A sex-cell from embryo B′ 26, in the region anterior to those illustrated in Figs. 14 and 15. The more medially placed cell is in the splanchnic mesoderm lateral to the gut. As this layer grows up over the gut to form the dorsal mesentery, the sex-cells will naturally be brought to lie in this region. × 700.

Fig. 17. Four sex-cells from the 6-day embryo B 30 in the mesentery dorsal to the gut. This shows in detail the region included in the rectangle in Text Fig. *C*. The extraordinary width of the mesentery at this stage is doubtless due in part to the presence of the sex-cells. × 420.

Fig. 18. This figure illustrates the extremes of the migration at this stage. No cell was found at any earlier stage than the one near the gut, none later than that ventral to the Wolffian duct. From embryo B 30. × 420.

Fig. 19. Showing the development of the gonads in the 13-day embryo B 42. The sex-cells are fixed in sac-like protuberances from the peritoneum. From this figure it is possible to obtain an idea of the effect produced by the rapid growth of the swim bladder, in literally pushing the gut and all related tissues ventrally. It is also interesting to observe that the peritoneal covering renders this cell dependent upon surrounding tissues for movement to its final position dorsal to the gut. × 420.

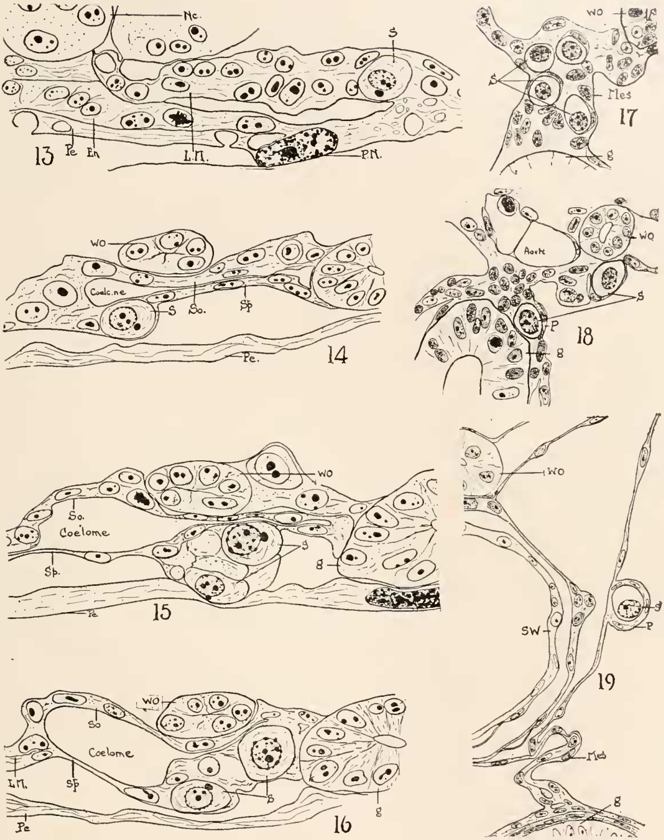

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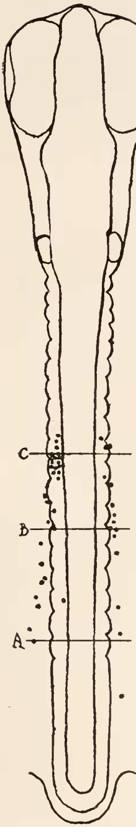
PLATE IV.

FIG. 20. A diagrammatic reproduction of embryo B′ 21 (46 hours) demonstrating the distribution of the sex-cells at this early stage. The variation in anterior-posterior development is very noticeable and is explained fully in the text. × 65.

FIG. 21. Reproduction of embryo B′ 26 (105 hours) constructed similarly to Fig. 20. Showing the positions of the sex-cells as being more medially placed than in the earlier stage. The cells on the right are not as far along with migration as those on the left; the former group migrated with the mesoderm and the latter followed the gut endoderm. × 65.



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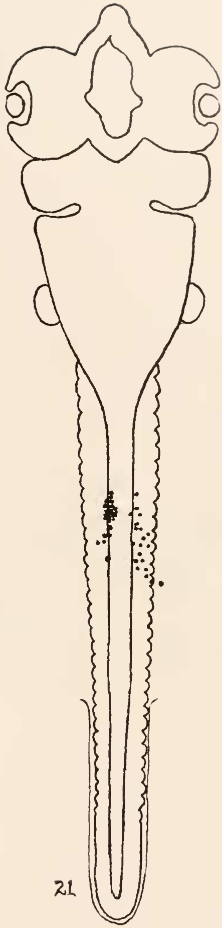

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SPERMATOGENESIS OF APHIDS; THE FATE OF THE SMALLER SECONDARY SPERMATOCYTE.

H. HONDA.

Contents.

I. Introduction.

It has been shown by Morgan and von Baehr that in aphids the primary spermatocyte divides unequally producing larger and smaller secondary spermatocytes. The larger secondary spermatocyte undergoes a second maturation division, and produces two equal-sized spermatids which transform into functional spermatozoa. The smaller secondary spermatocyte, which has received fewer chromosomes is said to degenerate. Only two similar spermatozoa, consequently, are formed from a primary spermatocyte.

Von Baehr (1909 and 1912) states that he very rarely observed the development of the smaller secondary spermatocyte to the prophase of the second maturation division, but that it does not divide. Stevens (1909) says that in a preparation which has unfortunately been lost, the anaphase of the smaller secondary spermatocyte was seen, and that such stage may also be distinguished among the degenerating spermatocytes. Morgan (1915) states: "The small cell is left with two chromosomes and a small amount of cytoplasm. It never divides again, and later degenerates. Stevens was inclined to think that the small cell may

sometimes show a division figure, which subsequently fades away, but I have never seen a case of this kind."

In *Macrosiphum ambrosiæ* and *Neothomasia populicola* I have observed the late telophase of the smaller secondary spermatocytes. In *Stomaphis yanois,* moreover, I have found that the smaller secondary spermatocytes divide and form equal-sized spermatids which are much smaller than the larger spermatids. These smaller spermatids develop and reach the sustentacular cells with the developed larger spermatids; they, however, fail to attach to the sustentacular cells. Thus their development ceases; they, therefore, do not fully transform into spermatozoa, but retrogress and form spherical cells, which attach themselves to the epithelium of the cysts of the testes. A further account of this will appear in the following pages.

The work on the spermatogenesis of *Stomaphis yanois* was done in the Tokyo Higher Normal College, and the work on the other aphids has been done in the University of Chciago. The writer's thanks are due to Prof. F. R. Lillie and Prof. S. Yamanouchi, who gave him many suggestions and much help. The writer also wishes to thank Prof. A. Oka and Prof. U. Takakura for their kindness during his stay in Tokyo. For the identification of the aphids the writer is indebted to Dr. A. L. Quaintance and Dr. A. C. Baker.

II. Methods.

Both the males and parthenogenetic females were fixed in either strong Flemming's, Zenker's or a mixture of absolute alcohol one part, acetic acid one part, and saturated aqueous solution of corrosive sublimate two parts. Sections were cut 3, 5 and 10 micra in thickness; most of them, however, were cut 5 micra thick. They were stained with Heidenhain's iron-hematoxylin followed by eosin or borax carmin.

III. Stomaphis yanois.

1. *Primary Spermatocyte.*

Figs. 1 and 2 show the primary spermatocyte prophase. In Fig. 1, one of the chromosomes is formed, and in Fig. 2, the proc-

ess of the formation of the chromosomes is almost finished. There are ten chromosomes, five larger and five smaller, in the equatorial plate of the first spermatocyte division, and they are connected with one another by linin threads as is shown in Figs. 4 and 5. The side view of the mitotic figure shows centrosomes of about the same size agreeing with von Baehr's observation on *Aphis saliceti* (Fig. 3).

In the anaphase unequal cell division is indicated. The larger and smaller daughter cells are connected by a bridge of cytoplasm, and elongated lagging chromosomes lie between the chromosomes passing to the daughter cells (Fig. 7). The lagging chromosomes do not show any tendency to go to the larger cell at this time, but after the nuclear membrane is formed, the lagging chromosomes enter the larger cell (Fig. 8). It is interesting to note that the size of the nuclear membrane is larger in the larger cell. The inequality of the size of the nuclei of the daughter cells, therefore, does not seem to be due to the unequal number of the chromosomes, but to an unequal quantity of cytoplasm. In a case where the two daughter cells were about equal the size of the nuclear membrane was about the same.

I have observed many cases in which the lagging chromosomes appear to be divided, but I doubt that this ever occurs. Morgan (1909) states: "That artificial conditions, such as handling or osmosis, might break such a delicate connection at this time is not at all improbable, and such an artificial result might give the impression that the accessory is actually divided. Moreover, if the bridge arches toward or away from the observer, the effect may be produced at certain focal levels of discontinuity between the ends of the lagging chromosomes, when none such exist."

The larger secondary spermatocyte receives eight divided and two lagging undivided chromosomes, and the smaller secondary spermatocyte receives eight divided chromosomes.

2. *Larger Secondary Spermatocyte.*

The larger secondary spermatocyte undergoes an equal second division without an intervening resting stage. The equatorial plate (Figs. 12 and 13) of the second division shows ten chro-

mosomes. In the first maturation division five of the ten chromosomes are larger and five of them are smaller, but in this case six are larger and four are smaller. The reason for this is discussed later on. As in the case of the first division, chromosomes are connected by linin threads. When the split chromosomes shift to the opposite poles interzonal fibers appear. In the first division the middle part of the two daughter cells becomes narrow and shows an appearance of a dumb-bell with the ends different in size. In this case, however, the middle part is broad, so that the interzonal fibers are separated (Fig. 15).

3. *Smaller Secondary Spermatocyte.*

The smaller secondary spermatocyte shows chromosomes in its nuclear cavity at the telophase of the first maturation division. It is not difficult to distinguish the smaller secondary spermatocyte as their diameter is hardly half that of the larger ones. The nucleus does not enter a resting stage. I have found in some cases two small bodies near the nuclear membrane (Fig. 9). These seem to be centrosomes, but I am unable to speak with certainty. The changes in preparation for the second division are similar to those of the larger spermatocyte.

The equatorial plate (Figs. 20 and 21) shows eight chromosomes as compared with ten chromosomes in the equatorial plate of the larger secondary spermatocyte. The cases which distinctly show eight chromosomes are rare; there can be little doubt, however, that this is the full number since there are ten chromosomes in the equatorial plate of the first maturation division, and two of them pass to the larger one as the lagging chromosomes. Four chromosomes are larger and the other four are smaller. There are five larger and five smaller chromosomes in the equatorial plate of the first division; the lagging chromosomes, therefore, must be a larger and a smaller chromosome. If all the chromosomes were to divide in the first division, five larger and five smaller chromosomes would appear in the equatorial plate of the second division. Two chromosomes, one larger and one smaller, lag and enter the larger cell without dividing. The smaller of the lagging chromosomes, consequently, becomes larger than the

other smaller chromosomes. This must be the reason why we see four smaller chromosomes in the larger secondary spermatocyte instead of five.

The side view of the metaphase of the smaller spermatocyte differs from that of the larger one in shape. It is more spindle-shaped (Fig. 22). Fibers are not seen distinctly in the preparations stained with iron hematoxylin. The two stained bodies on both sides of the chromosomes in the equatorial plate might be the centrosomes (Fig. 23). There are cases which show separated chromosomes, and cases which show massed chromosomes (Figs. 23–25). So far as my observation goes, in most cases the chromosomes seem to fuse soon after their splitting. The telophase does not show distinctly the interzonal fibers as in the case of the larger cell. Two equal smaller spermatids are produced after the division.

4. *Smaller Spermatid.*

The germ cells of each cyst of the testes are generally in about the same stage. When the spermatids are young the cysts are spherical in shape, but they elongate during the development of the spermatids. The young smaller spermatids (Fig. 28) have condensed nuclei, but the larger spermatids (Fig. 18) between which they lie have vesicular nuclei. These smaller and larger spermatids are seen all through the cyst. I have examined many cases in order to see whether the polarities of the larger and smaller spermatids are established with relation to the epithelium or not. Most of the young larger and smaller spermatids, which are seen near the epithelium, develop their tails toward the center of the cyst, but some of them may develop along the epithelium or develop their tails toward the epithelium. Those in the central part do not show any definite orientation, and in extreme cases spermatids existing side by side may show opposite directions. In cysts in which the larger spermatids are developed to the stage shown in Fig. 18, the orientation of the larger and smaller spermatids remains unchanged. In a little later stage, however, all the larger and smaller spermatids begin to orient in the same direction, and when the larger spermatids develop to the stage shown in Fig. 19, all are oriented in the same direction.

There must be an interaction, probably chemical, between the sustentacular cells and the larger and smaller spermatids. The larger and smaller spermatids in the outer part opposite the sustentacular cells and in the central part of the cyst generally move among the tails of the other spermatids toward the sustentacular cells, but those in the other parts move toward sustentacular cells along the epithelium.

Developed smaller spermatids (Fig. 31) are seen among the larger spermatids near the sustentacular cells, and do not show any inferiority to the larger spermatids in moving toward the cells. Before the nucleus of the larger spermatid shows marked differentiation the smaller spermatids have retreated a little towards the interior. In other words, well developed smaller spermatids approach towards the sustentacular cells, but do not attach to them. I have examined many smaller spermatids in order to see whether they develop apical parts. Figure 30 shows a developing smaller spermatid, which has a cone-shaped apical part. There is a developed smaller spermatid, which seems to have a well-developed apical part, but we cannot distinctly observe since it is seen in close contact with the tails of the larger spermatids. In most of the smaller spermatids, which have elongated tails, I have not, however, observed developed apical parts.

As to the interpretation of the cells identified as smaller spermatids, may they not be degenerating larger spermatids? So far as my observation goes the larger spermatids rarely degenerate; moreover, it is not hard to distinguish degenerating young larger spermatids from the smaller spermatids, since the former are not only much larger than the latter, but the nucleus of the larger spermatid becomes vesicular while the nucleus of the smaller spermatid is condensed. If the larger spermatids developed to the stage shown in Fig. 19 begin to degenerate, we can recognize them by the difference in the state of the nuclei. If the almost fully developed spermatids begin to degenerate, it is quite easy to tell them from the smaller spermatids, since they have very slender nuclei and the smaller spermatids, which are seen in the same cyst with them, have spherical nuclei. If degeneration of the larger spermatids should occur at the stage in which they

have condensed ovoid nuclei which elongate later, the criterion by which to distinguish them is their position, since when they have developed to such a stage, the smaller spermatids with condensed spherical nuclei have already left the epithelium.

The metaphase of the smaller secondary spermatocytes are seen among those of the larger secondary ones; I think, therefore, there is no doubt that the smaller secondary spermatocytes undergo the second division. More developed larger spermatids are seen with more developed smaller spermatids in the same cyst. We may conclude from these observations that the smaller spermatids develop with the larger spermatids.

I have observed cases where the larger and smaller spermatids are seen in the central part of the cyst, while the majority of spermatids have already reached the sustentacular cells. Such larger and smaller spermatids might fail to reach the sustentacular cells, since they have to move among the spermatids. The examination of the later stages, however, has shown that they succeed in reaching the sustentacular cells.

Figure 32 shows a smaller spermatid which is abnormally big and has a distinct axial filament. Ordinarily the smaller spermatids elongate similarly, but are more delicate. One of the most developed smaller spermatids is shown in Fig. 33. In such a stage their development comes to a standstill, and they begin to retrogress. They gradually retreat toward the tails of the larger spermatids. Their nuclei which are deeply stained with iron hematoxylin are seen among the tails of the larger spermatids in a somewhat regular position. Finally they pass out to the cavity of the cyst.

The smaller spermatids fail in attaching to the sustentacular cells; they cannot, consequently, get material for their further development. They have to live on their own substance. Their tails become shorter, and the cytoplasm around the nucleus increases (Fig. 34).

The forms shown in Fig. 35 are seen near the tail of the fully developed functional spermatozoa in the cavity of the cyst. We do not see such spermatids in the cavities of the cysts at the younger stages. These smaller spermatids still have elongated tails, but later transform into spherical cells which have a distinct cell

membrane (Fig. 39) and show a tendency to fuse with each other. There are some cells which have two or more condensed nuclei. These seem to be the products of the fusion of two or more smaller spermatids. Some of the retrogressed cells of the smaller spermatids attach to the epithelium, and on these cells other cells attach themselves; thus they form layers as shown in Fig. 38. In other cases they are irregularly attached to the epithelium. When they attach themselves to each other they show a polygonal shape.

A, b and *c* in Fig. 38 are parts of adjacent cysts, where fully developed spermatozoa occur though not shown in the figure. The cells occurring between the cysts are the retrogressed smaller spermatids produced in the cyst *c*, and the epithelium proper is very thin as seen between cysts *a* and *b*. As we see in the figure these cells are not equal in size. In some of them the nuclei are broken up and their fragments are seen scattered throughout the cells. The others still show condensed spherical nuclei. As stated already the larger spermatids rarely degenerate. These larger spermatids may become like the cells just mentioned. Though degenerating larger spermatids mingle among these cells, there is no criterion by which they may be distinguished from retrogressed cells of the smaller spermatids.

Some of these cells may be absorbed by the epithelial cells, but how far the absorption proceeds is at present undetermined. When these cells attach to the epithelium the functional spermatozoa are already fully developed. Afterwards the wall of the cyst ruptures, and these cells being deprived of their connection with the testis are destined to disappear. It is possible that they are extruded from the testis along with the spermatozoa. I have observed epithelial cells of the cyst and retrogressed cells of the smaller spermatids in some of the vasa deferentia. The sections of the testes of the old males show remarkable changes. Their walls are thickened and neither spermatozoa nor the cysts, which fill the young testes, can be seen.

IV. Neothomasia populicola and Macrosiphum ambrosiæ.

The testes of embryos of *Neothomasia populicola* and *Macrosiphum ambrosia* are in the early stages, but those of larvæ are

suitable for the purpose of studying the spermatocyte divisions. As is the case in other aphids, the primary spermatocytes of these aphids divide unequally, and the anaphase shows the lagging chromosomes. I have found in these aphids telophases of the second maturation division of the smaller secondary spermatocyte, but have observed no developing smaller spermatid; we may, therefore, conclude that the smaller secondary spermatocytes and the smaller spermatids of these aphids degenerate as in the cases of the aphids studied by Morgan, von Baehr and Stevens. In *Macrosiphum ambrosia* I observed cases in which all smaller secondary spermatocytes seemed to be dividing, but I will conclude in a succeeding paper whether all the smaller secondary spermatocytes divide or not.

In the cyst, where larger spermatids are already attached to the sustentacular cells, there are seen spermatids which look like the smaller spermatids of *Stomaphis yanois.* As stated above, since no development of the smaller spermatids was observed, they must be larger spermatids. In slightly younger cysts some spermatids are seen among the developed tails of other larger spermatids, which are about to attach to the sustentacular cells. Such spermatids probably have no chance of reaching the cells. I have found cases in which the larger spermatids are already attached to the cells, but some spermatids are seen among the ends of the tails of the larger spermatids. In other cysts spermatids with condensed nuclei are seen apart from the sustentacular cells, while others are attached to them.

As in the case of *Stomaphis yanois* young spermatids of these aphids change their orientation to the same direction; some spermatids, therefore, move to the sustentacular cells across the whole diameter of the cyst or reach the cells moving along the epithelium. If they move to the sustentacular cells along the epithelium, as most of the spermatids do, they may lose the chance to become attached to them. Developed spermatids have been found by the side of spermatids which are attached to the sustentacular cells and are developing. They were probably prevented from reaching the cells by other spermatids, and their development came to a standstill; they, consequently, show younger stages than the spermatids which are attached to the

cells. Since many spermatids are produced in the cysts, if they move to the sustentacular cells through the tails of other spermatids, they meet much resistance; they, therefore, might be unable to reach the cells.

The most conspicuous difference between the case of *Stomaphis yanois* and that of these aphids is the position of the retrogressing spermatids. In the former case the smaller spermatids approach the sustentacular cells, and then gradually retreat toward the tails of the larger spermatids; their position, consequently, is regular, having a relation to the development of the larger spermatids. In the latter case, however, the position of the retrogressing spermatids is irregular.

As in the case of *Stomaphis yanois* retrogressed spherical cells are seen in the cyst with fully developed spermatozoa. These cells attach themselves to the epithelium of the cysts and have the same fate as the retrogressed cells of the smaller spermatids of *Stomaphis yanois.*

V. Review.

According to Meves and others, one of the secondary spermatocytes of the honey bee is much smaller than the other, and receives no chromosomes; it, consequently, degenerates after some time. The larger secondary spermatocyte, moreover, divides unequally in the second spermatocyte division. The chromosomes divide this time, and there are produced larger and smaller spermatids. The larger spermatids differentiate into functional spermatozoa. The smaller spermatids also undergo some differentiation which, however, comes to a standstill at a late stage and then they degenerate without transforming into functional spermatozoa. The smaller spermatid of *Stomaphis yanois* resembles that of the honey bee in some respects. Both of them are much smaller than the larger spermatids, but judging from the Meves' drawings, the difference in size between the larger and the smaller spermatids is greater in the honey bee than in the aphid. They both develop to some extent, but do not transform into functional spermatozoa. Meves does not state what kind of changes occurs in the degenerating smaller spermatids of the honey bee; I am, therefore, unable to compare their later stages with those of the smaller spermatids of *Stomaphis yanois.*

The most conspicuous difference between the smaller spermatids of the honey bee and this aphid is seen in the nuclei. The nucleus of the smaller spermatid of the honey bee returns to a resting stage, and differentiates similar to that of the larger spermatid. The nucleus of the smaller spermatid of this aphid, however, becomes condensed after the second spermatocyte division, and remains in the same state, although the cytoplasm shows changes similar to those of the larger spermatid. This may be caused by the absence of the lagging chromosomes in the smaller spermatids, while in the honey bee the smaller spermatids have the same number of chromosomes as the larger spermatids.

Whitney (1918) mentions that the normal and rudimentary spermatozoa have been found in considerable number of rotifers. In his paper of 1917 he says that the functional spermatozoa are identical in their power of determining the sex of the individual that develops from a fertilized egg, since after a functional spermatozoon has fertilized a parthenogenic male egg, the egg always develops into a female individual.

In the case of these rotifers, according to Whitney, the chromosomes divide in the first spermatocyte division. One half of the secondary spermatocytes divide and form the normal spermatids. The remaining half of the secondary spermatocytes, contrary to the case of the smaller secondary spermatocyte of *Stomaphis yanois,* do not divide, but develop directly into the degenerate spermatozoa. The spermatocytes destined to degenerate are smaller than the others, and their development into the complete rudimentary spermatozoa is strikingly different from the development of the normal spermatids.

Whitney ('18) says that as all the fertilized eggs in both phylloxerans and rotifers develop into female young, it seems safe to conclude, as Morgan has already concluded, that the degenerate sperm cells are the male-determining ones and that the normal sperm cells are the female-determining ones.

Stevens (1905) found many degenerate spermatozoa in *Blattella germanica.* She states that the distribution and varying number of these degenerate spermatozoa make it impossible to interpret their condition as due to the absence of the accessory chromosome as Miss Wallace does in the spider, and that the only

probable explanation seems to lie in the imperfect mitosis. She detected no evidence of degeneracy among the young spermatids.

VI. Summary.

1. In *Stomaphis yanois* the smaller secondary spermatocytes divide, and develop to some extent, but retrogress to spherical cells.

2. In *Neothomasia populicola* and *Macrosiphum ambrosia*, cases of division of the smaller secondary spermatocytes were found, but no developing smaller spermatids were observed.

3. In *Neothomasia populicola* and *Macrosiphum ambrosia* spherical cells like those in *Stomaphis yanois* were found in the cysts containing spermatozoa. These were identified as retrogressed larger spermatids.

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EXPLANATION OF PLATES.

All of the drawings were made with the aid of camera lucida. Figs. 1 to 15 were drawn with a Leitz 1/16 oil immersion objective and a Zeiss compensating ocular 18. Figs. 16 to 37, except Fig. 33, were drawn with a Leitz 1/16 oil immersion objective and a Leitz ocular 5. Fig. 33 was drawn with a Leitz 1/16 oil immersion and a Leitz ocular 4. Fig. 38 was drawn with a Leitz 1/16 oil immersion objective and a Leitz ocular 3. All figures from *Stomaphis yanois.*

Plate I.

Figs. 1 and 2. Primary spermatocytes, prophase.

Figs. 3, 4, 5 and 6. Primary spermatocytes, metaphase.

Fig. 7. Primary spermatocyte, anaphase.

Figs. 8 and 9. Primary spermatocytes, telophase.

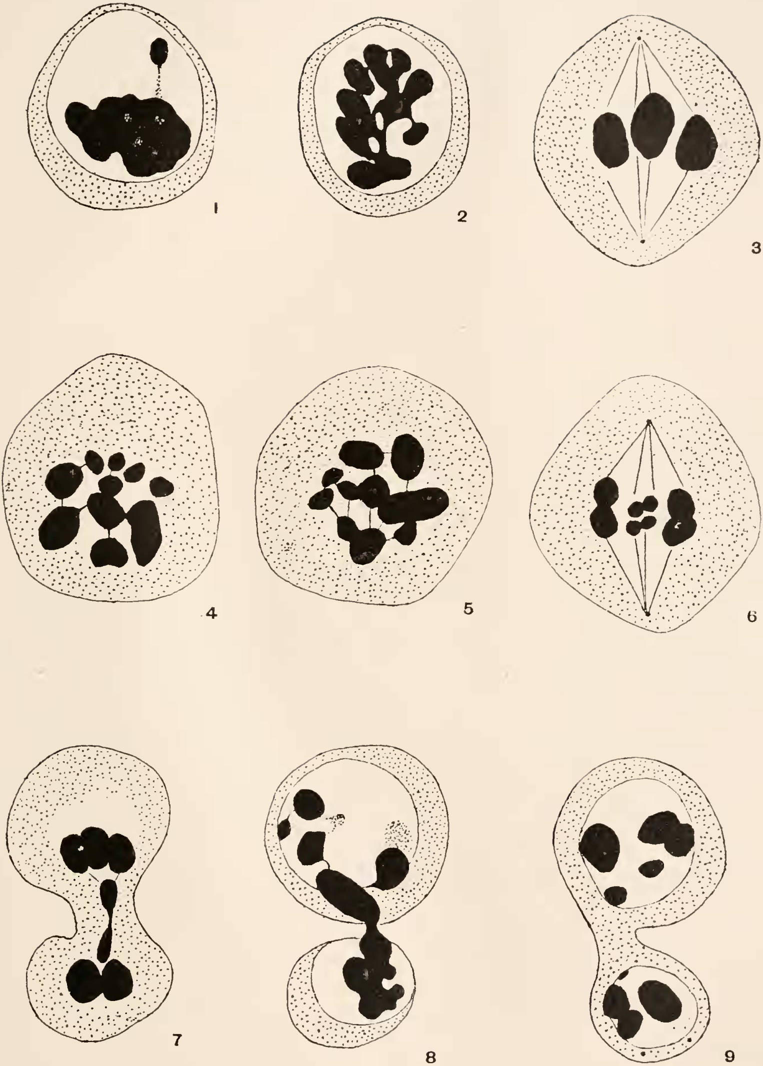

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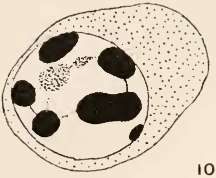
PLATE II.

FIG. 10. Larger secondary spermatocytes, prophase.
FIGS. 11, 12 AND 13. Larger secondary spermatocyte, metaphase.
FIGS. 14 AND 15. Larger secondary spermatocytes, anaphase.
FIG. 16. Larger secondary spermatocyte, telophase.
FIGS. 17, 18 AND 19. Larger spermatids.



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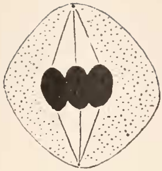

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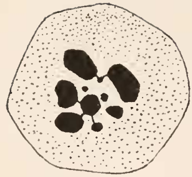

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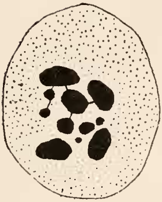

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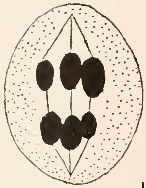

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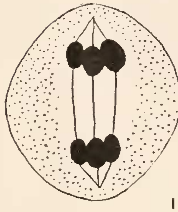

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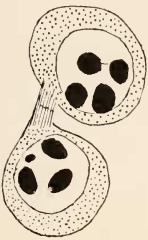

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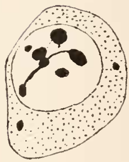

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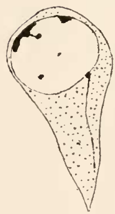

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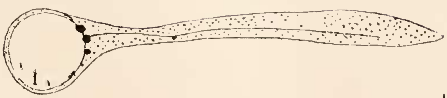

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PLATE III.

FIGS. 20, 21, 22, 23 AND 24. Smaller secondary spermatocytes, metaphase.
FIGS. 25 AND 26. Smaller secondary spermatocytes, anaphase.
FIG. 27. Smaller secondary spermatocyte, telophase.
FIGS. 28, 29, 30 AND 31. Smaller spermatids.


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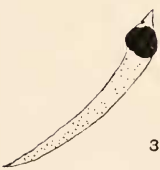
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Plate IV.

Figs. 32 and 33. Smaller spermatids.
Figs. 34, 35 and 36. Retrogressing smaller spermatids.
Fig. 37. Retrogressed cell of the smaller spermatid.
Fig. 38. Layers of the retrogressed cells.

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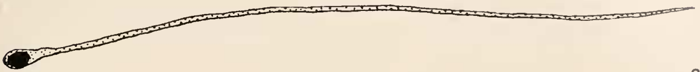

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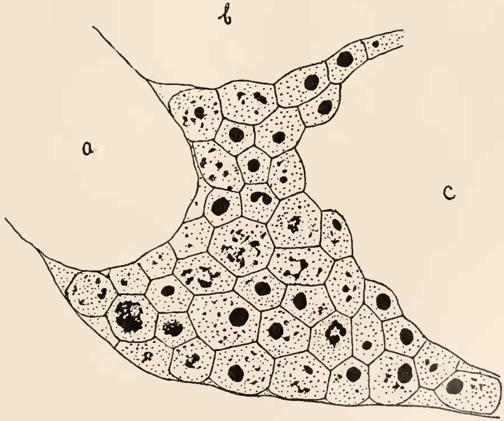

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